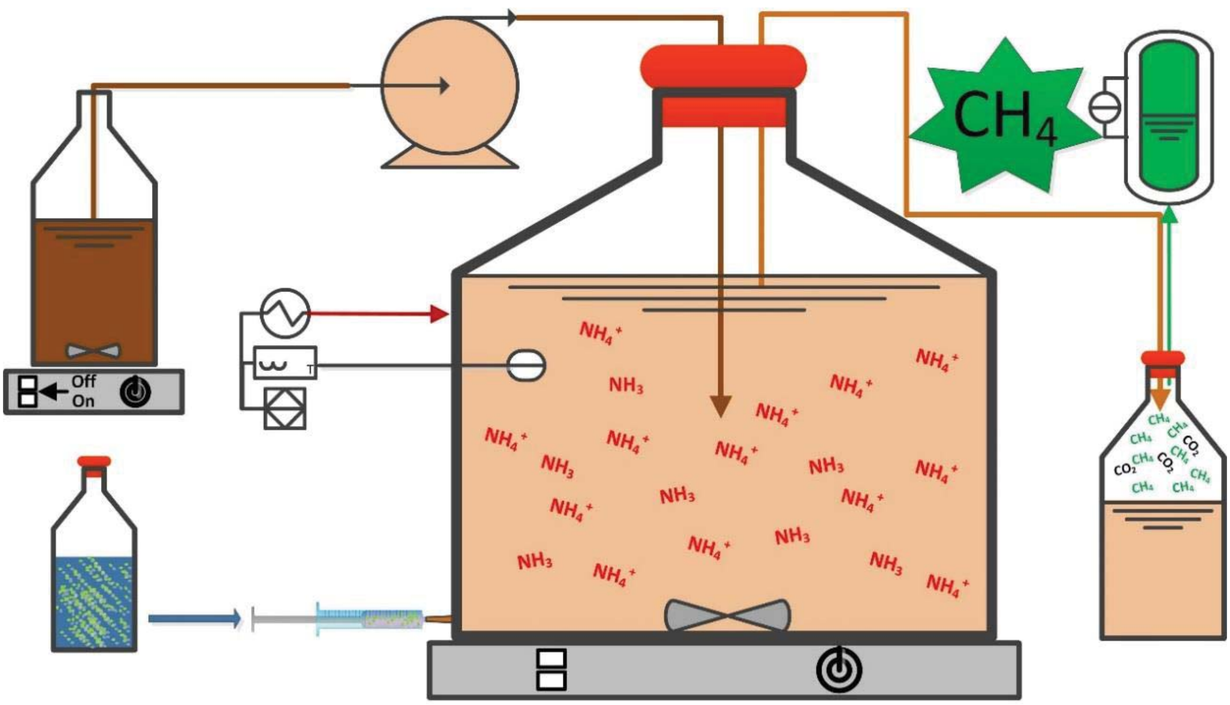
Innovative bioaugmentation strategies to tackle ammonia inhibition in anaerobic digestion process-MicrobStopNH$_3$

Final Project Report

Ioannis A. Fotidis and Irini Angelidaki



October 2019

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Cover photo: The bioaugmentation concept by Ioannis A. Fotidis

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Preface

All the MicrobStopNH$_3$ project research activities took place in the Department of Environmental Engineering of the Technical University of Denmark by the Bioenergy Group. During the 50 months of the project, together with the two authors of the current report, three PhD and 16 MSc students, as well as the director and the staff members of Lemvig biogasanlæg Amba, have participated in the project activities. We would like to thank all the people who contributed to the completion of the MicrobStopNH$_3$ project.

Finally, we would like to thank the Energinet.dk, which under the project framework ForskEL "Innovative bioaugmentation strategies to tackle ammonia inhibition in anaerobic digestion process-MicrobStopNH$_3$" (program no. 2015-12327), has funded all the project activities.

Kgs. Lyngby, October 2019

Ioannis A. Fotidis	Irini Angelidaki
Associate Professor	Professor

Content

Final report

1.1 Project details

Project title	Innovative bioaugmentation strategies to tackle ammonia inhibition in anaerobic digestion process
Project identification (program abbrev. and file)	MicrobStopNH_3-12327
Name of the programme which has funded the project	ForskEL
Project managing company/institution (name and address)	DTU Environment, Bygningstorvet Bygning 115, 2800 Kgs. Lyngby
Project partners	Lemvig biogasanlæg Amba
CVR (central business register)	30060946
Date for submission	30-09-2019

1.2 Short description of project objective and results

1.2.1 English project description

The MicrobStopNH_3 project aimed to develop the bioaugmentation of ammonia tolerant methanogenic consortia, as a new technical approach to alleviate the ammonia inhibitory effect in continuous anaerobic digesters fed with ammonia-rich substrates. The project activities included lab-scale experiments, a pilot-scale assessment and economic and environmental feasibility analyses of the bioaugmentation process. The results of the project showed that it is possible and environmentally and economically beneficial to use bioaugmentation of pure or mixed methanogenic cultures in ammonia inhibited continuous reactors, to improve instantly the methane production between 13 and 40%, under both mesophilic and thermophilic conditions.

1.2.2 Danish project description

MicrobStopNH_3 projektet sigtede mod at udvikle bio-augmentering (biologisk berigelse med bestemte mikrooorganismer) af ammoniak-tolerante metanogene grupper som en ny metode for at afværge den ammoniakhæmmende virkning i kontinuerlige anaerobe reaktorer der behandler ammoniakrige substrater. Projektaktiviteterne laboratorie- og pilotskalaforsøg og økonomisk og miljømæssige analyser af processen. Resultaterne af projektet viste, at det er teknisk muligt samt miljømæssigt fordelagtigt at anvende bio-aubmentering med rene eller blandede metanogene kulturer i ammoniak-hæmmede biogas reaktorer. Efter tilsægning af mikrorganismerne blev metan produktionen straks forbedret med ca. 13 - 40%, under såvel mesofile som termofile forhold.

1.3 Executive summary

Every year the agricultural and the food industrial sectors produce vast amounts of ammonia-rich organic wastes, which require the development of a specific, efficient and sustainable treatment. One of the most promising and effective could be anaerobic digestion as it provides energy (methane) and a digestate with high nutrient levels, which can be used as fertilizer. Unfortunately, ammonia-rich substrates are well known to inhibit this process; it is estimated that many full-scale biogas reactors in Denmark and worldwide are seriously affected by ammonia toxicity constantly loosing up to 1/3 of their methane producing potential. It has been widely proven that ammonia is mainly inhibiting the aceticlastic methanogenic pathway, while the syntrophic acetate oxidation pathway followed by hydrogenotrophic methanogenesis is more robust to ammonia toxicity. It is therefore reasonable to assume that the preferential use of ammonia tolerant hydrogenotrophic methanogenic consortia could provide a new solution to alleviate the ammonia inhibitory effect in AD process. The MicrobStopNH$_3$ project proposed bioaugmentation as a new technical approach to establish these ammonia tolerant consortia in continuous digesters operating under ammonia inhibitory pressure. Based on that, during the project this new technology (bioaugmentation) in biogas reactors for overcoming/avoiding ammonia inhibition was developed. The project besides many lab-scale experiments included a pilot-scale assessment and economic and environmental feasibility analyses of the bioaugmentation process. The main results of the project included:

- The identification and enrichment of more than ten (mesophilic and thermophilic) ammonia tolerant methanogenic cultures that subsequently were used as bioaugmentation inocula.
- The assessment of pure and complex ammonia tolerant methanogenic communities as bioaugmentation inocula and the identification of their strengths and weaknesses under different operation conditions.
- The identification of the crucial role of hydrogenotrophic methanogens in the success of the bioaugmentation process under extreme ammonia conditions and the proposal of the biochemical mechanisms that dictate the process.
- The definition of the "critical biomass" (minimum amount) of at least 2% v/v bioaugmentation inoculum for a successful bioaugmentation process.
- The immediate improvement of methane production between 13 and 40%, in lab-scale continuous reactors, fed with ammonia-rich substrates, after bioaugmentation with pure or mixed cultures at mesophilic and thermophilic conditions.
- The identification of the optimal environmental and operational conditions in order to enhance the effect of the identified inocula on ammonia-rich substrates.
- The development of a realistic strategy for the upscale of bioaugmentation to a pilot-scale reactor, which yielded to an immediate methane production improvement of ≈40%, compared to the inhibited period.
- The clearly demonstrated positive outlook on the climate change and emissions avoidance, due to substituted energy production in both assessed counties (i.e. Denmark and Italy), of the bioaugmentation system analyses.

We expect that the results of the MicrobStopNH$_3$ project can be further utilised in full-scale reactors that are using, or they will use ammonia-rich substrates. In order to achieve this, the readiness level of the developed technology must be moved pass the pilot-scale assessment and create a commercially applicable process. This commercially available bioaugmentation technology is the next major goal of the researchers involved in the MicrobStopNH$_3$ project as well as other researchers around the world that they have included bioaugmentation in their research activities.

1.4 Project objectives

MicrobStopNH3 was aiming to develop an innovative, efficient and robust bioaugmentation technology to counteract ammonia toxicity effect in anaerobic digesters, enhancing biogas production up to 35%. The project had the following sub-objectives:

- Identify the best ammonia tolerant methanogenic consortia for a robust bioaugmentation process under mesophilic and thermophilic conditions.
- Define the minimum necessary amount of ammonia tolerant methanogenic inoculum ("critical biomass") for successful bioaugmentation in continuous stirred tank reactors (CSTR).
- Define the operational and environmental parameters to enhance the bioaugmentation efficiency of the identified inocula.
- Validate the developed bioaugmentation protocol and applying it to a pilot-scale reactor suffering from ammonia toxicity.

As it is analytically presented in the following sections (i.e. 1.5, 1.6, 1.7 and Annex) of the current report, all the main objectives of the project were completed successfully. There was only one major (partially unexpected) problem during the completion of the project, which was the technical preparation and the controlled operation of the pilot-scale reactor. This led to a 14 months extension of the project's timeframe, which allowed us to address all the technical problems of the pilot-scale reactor. After the pilot-scale reactor became operational, the optimum bioaugmentation protocol that was developed during the previous project activities, was applied. Overall, the different WPs of the project have been completed and as shown in the Gantt diagram (Table 2).

Table 2. *MicrobeStopNH$_3$ Gantt diagram*

Activities	**2015**	**2016**				**2017**				**2018**				**2019**		
	Q4	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**	**Q3**
Ammonia tolerant methanogenic consortia																
Bioaugmentation process development for continuous fully mixed reactors																
Pilot-scale verification																
System analysis																

The milestones of the project were:

M1 Robust mesophilic and thermophilic ammonia tolerant bioaugmentation inocula.
M2 Report for optimal bioaugmentation conditions (inoculum, temperature, VFA, ammonia, OLR, HRT).
M3 A complete bioaugmentation protocol to establish ammonia tolerant methanogens in large-scale CSTR reactors.
M4 Evaluation report of the bioaugmentation process technology.

All the four milestones that were described in the project have been fulfilled successfully. The specific documented project activity that corresponds to each one of the milestones are indicated with the milestone abbreviation (M1-M4) in the students (subsection 1.5.6) and dissemination activities (section 1.6) of the project.

The deliverables of the project (i.e. 4-5 ISI publications and 3 conference contributions) were exceeded by at least 3-fold since 12 published ISI papers and 12 conference contributions (section 1.6, dissemination activities) were produced. Additionally, 6 ISI papers with results from the project activities are currently under preparation.

Finally, it must be mentioned that, all the potential risks that were identified in the project proposal (such as: a) The ability to use new mixed ammonia tolerant methanogens as mesophilic and thermophilic bioaugmentation inocula. b) The identification of the "critical biomass" of ammonia tolerant methanogenic inoculum for successful bioaugmentation. c) The identification of the optimal environmental conditions for successful bioaugmentation under thermophilic conditions. d) The successful bioaugmentation of continuous reactors under thermophilic conditions), were addressed successfully during the project, and did not affect the completion of the project.

1.5 Project results and dissemination of results

The MicrobStopNH$_3$ project had four main research activities and their most significant results are presented in the current section. Nonetheless, since it was not possible to present analytically all the project activities and their results, a brief presentation of all the documented experimental activities that performed during the project are included in the "1.6 Dissemination activities" and in the "Annex" sections of the current report.

It must be stated that this project was not aiming at any commercial results since it was a novel technology development and thus no turnover, exports or employment was part of the results. However, if the developed and verified MicrobStopNH$_3$ technology is being used in full-scale conditions, we expect great economic benefits for the existing biogas sector, which will lead to more biogas plants to be built and thus more people to be employed.

1.5.1 Ammonia tolerant methanogenic consortia

The main aim of the current research activity was to create and identify ammonia tolerant mesophilic and thermophilic methanogenic consortia that could be used as bioaugmentation inocula. In order to fulfil this aim, we had to overcome the lack of appropriate technologies to create ammonia tolerant methanogenic consortia. Therefore, we assessed three different cultivation methods (i.e. batch, fed-batch and CSTR) to acclimatise methanogenic consortia to high ammonia levels. In order to evaluate the effect of temperature in the different acclimation processes both mesophilic (37°C) and thermophilic (53°C) inocula were used. To evaluate the three acclimation processes methane production efficiency, incubation time, TAN/FAN (total ammonium nitrogen/free ammonia nitrogen) levels achieved and methanogenic activity, were used as criteria. Finally, both stepwise and direct exposure of methanogens to ammonia during batch cultivation, were tested during the batch experimental assay (Figure 1).

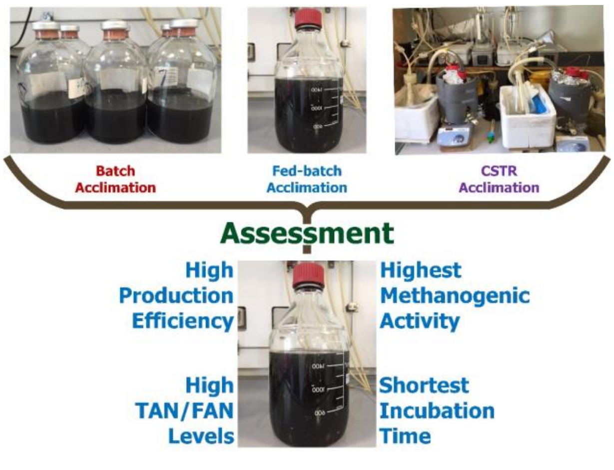

Figure 1. *The overall all acclimatization concept.*

The methane production in all batch reactors reached the theoretical value through different incubation times (Figure 2). During direct-exposure acclimation method, the incubation time was prolonged alongside the ammonia levels, due to the longer lag phases. The stepwise acclimation method shortened the incubation time within each individual ammonia level both under mesophilic and thermophilic condition compared to direct exposure. However, even though the shorter incubation time within each individual acclimation step, the stepwise exposure still took more time to acclimatise the consortia to high ammonia levels than the direct method. The incubation time for the highest ammonia levels was 78 and 91 days (Fig. 2A and Fig. 2B) under direct-exposure for mesophilic and thermophilic condition, respectively, while it was 125 and 158 days under stepwise-exposure (Fig. 2C and Fig. 2D). This was probably due to the accumulation of lag phases during every single step of the stepwise exposure. On the other hand, it was reported that direct-exposure, results in higher diversity of methanogens compared to stepwise acclimation, which may provide a better chance for the methanogens to adapt to the high ammonia environment. Therefore, based on the results from the current study, the direct-exposure acclimation method seems to be preferable than the stepwise-exposure, with respect to methane production and especially the incubation time.

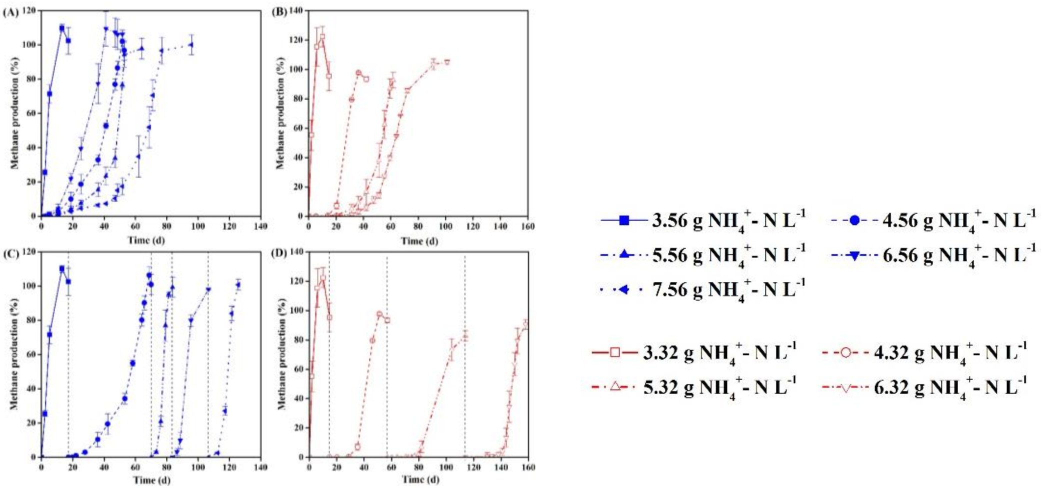

Figure 2. *Methane production of batch acclimation method, (A) direct-exposure a mesophilic condition, (B) direct-exposure at thermophilic condition, (C) stepwise-exposure at mesophilic condition, (D) stepwise-exposure at thermophilic condition.*

Methane production for the fed-batch reactors followed the theoretical methane production with only small fluctuations and finally reached a high TAN concentration of 6.56 and 6.32 g NH_4^+-N L^{-1} for mesophilic and thermophilic conditions, respectively (Figure 3). Even though a gas production delay was seen at 4.32 g NH_4^+-N L^{-1}, the reactor recovered immediately and at the end of the experiment, methane production was more than 83% of the theoretical expected for both fed-batch reactors. This result clearly indicated a stable AD process without any ammonia inhibition. Interestingly, the final FAN concentration was more than 1600 mg NH_3-N L^{-1}in the thermophilic reactor. The successful acclimatisation to these extremely high FAN levels at such a short time frame (64 days) has never been reported before. The initial inocula were successfully acclimatised to 6.56 and 6.32g NH_4^+-N L^{-1} under mesophilic and thermophilic condition, respectively. During the whole process, no instability due to high ammonia was observed.

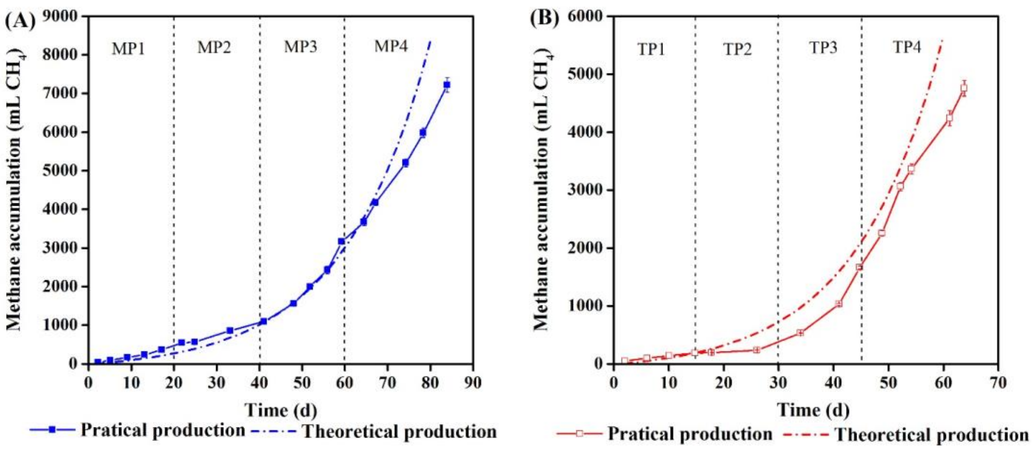

Figure 3. *Methane accumulation at (A) mesophilic and (B) thermophilic fed-batch reactors.*

Both CSTR reactors did not pass the second acclimation step (4.56 g NH_4^+-N L^{-1} for mesophilic and 4.32 g NH_4^+-N L^{-1} for thermophilic) due to strong ammonia inhibition. An ammonia induced "inhibited steady state" was established, with a methane yield of around 30% of the theoretical, until the end of the experiment (Figure 4). The low methane yield indicated that methanogens were experiencing strong inhibition from the ammonia. The main reason for the failure of both CSTR reactors could be the washout of methanogenic communities, which resulted in the loss of ammonia tolerant methanogens that were in low abundance in the initial inocula. Therefore, it can be concluded that CSTR reactors, even if they are successful, they are not suitable for the acclimation of ammonia-tolerant methanogenic consortia in a realistic timeframe that will allow them to be used as bioaugmentation inocula. However, if time is not an issue, then it could be possible to use longer HRTs and slower exposure to higher ammonia levels to efficient acclimatise methanogenic inocula, as we have also shown in other acclimatisation experiments contacted during the MicrobStopNH3 project.

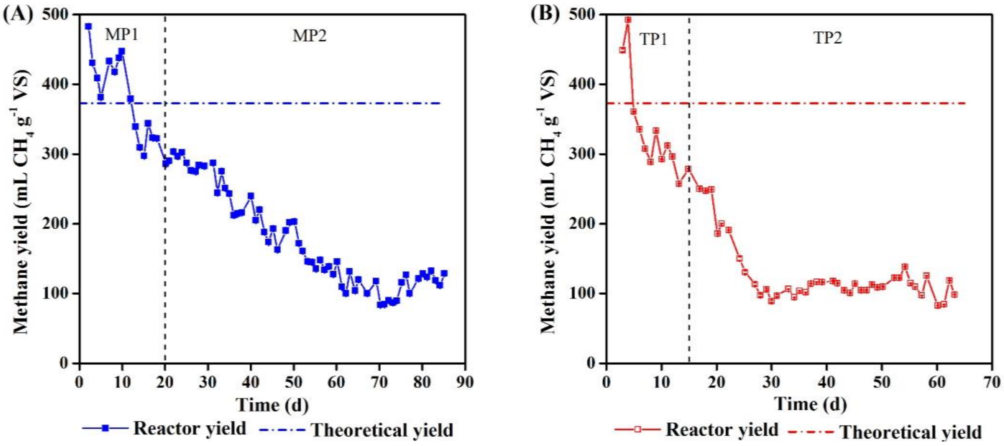

Figure 4. *Methane yield at (A) mesophilic and (B) thermophilic CSTR reactors*

The activity test (Figure 5) showed that hydrogenotrophic methanogens were significantly more active than aceticlastic methanogens among most of the acclimation methods at higher ammonia levels.

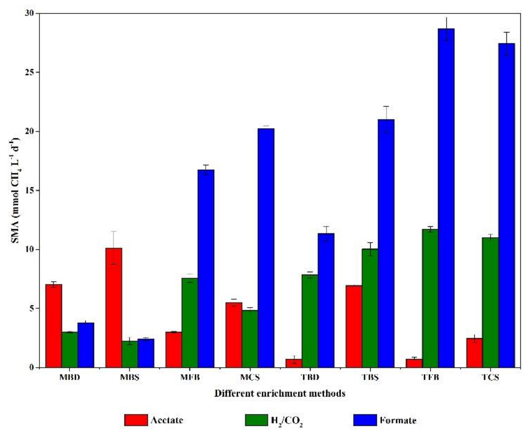

Figure 5. *SMA test results of mesophilic batch direct-exposure (MBD), mesophilic batch stepwise-exposure (MBS), mesophilic fed-batch (MFB), mesophilic CSTR (MCS), thermophilic batch direct-exposure (TBD), thermophilic batch stepwise-exposure (TBS), thermophilic fed-batch (TFB) and thermophilic CSTR (TCS).*

Aceticlastic activity was higher only in mesophilic batch reactors, with no significant difference between direct and stepwise acclimation approach. This therefore means that aceticlastic had higher growth rates compared to the hydrogenotrophic methanogens, under the same growth conditions. Thus, the higher aceticlastic activity could be explained by the low FAN levels (210 mg NH_3-N L^{-1}) during the mesophilic batch acclimation that did not affect the growth rates of the aceticlastic methanogens. Thus, based on the activity test results and the FAN levels, hydrogenotrophic methanogens had higher activity under high FAN levels (>540 mg NH_3-N L^{-1}), while aceticlastic methanogens were more active at low FAN levels (<210 mg NH_3-N L^{-1}).

Finally, the evaluation (Table 2) of the three different acclimatization methods, based on the chosen assessment criteria (i.e. methanogenic activity, methane production efficiency, incubation time and ammonia levels achieved), shown that fed-batch was the best acclimation method compared to batch and CSTR methods, under mesophilic and thermophilic conditions. In the current acclimatization experiment, the CSTR acclimation method yielded only 30% production efficiency compared to the theoretical, while at the same time more than 83% was achieved in batch and fed-batch reactors.

Table 2. *Comparison between the three different acclimation methods*

	Mesophilic inoculum				Thermophilic inoculum			
	Batch direct	**Batch stepwise**	**Fed-batch**	**CSTR**	**Batch direct**	**Batch stepwise**	**Fed-batch**	**CSTR**
Highest TAN (g NH_4^+-N L^{-1})	7.56	7.56	6.56	4.56	6.32	6.32	6.32	4.32
Highest FAN (mg NH_3 L^{-1})	208	181	549	490	614	542	1633	1425
Incubation time (d)	78	125	84	-	91	158	64	-
Methanogenic activity (mmol CH_4 L^{-1} d^{-1})	7.04	10.13	16.75	20.21	11.33	20.99	28.68	27.43
Production efficiency (%)	100	100	86.5	32	100	100	83.9	30

Among the other acclimation methods, the shortest incubation period was achieved together with the highest methanogenic activity and the highest FAN levels, with fed-batch reactors for both mesophilic and thermophilic conditions. Specifically, the FAN levels and the maximum methanogenic activity of the mesophilic fed-batch were 164% and 138% higher than batch direct-exposure method, respectively. The thermophilic fed-batch acclimation method had the highest FAN levels and activity, but also had more than 40% shorter incubation time compared to batch acclimation methods. Therefore, based on the comparison of incubation time, methanogenic activity, production efficiency and ammonia levels between the different acclimation methods, it seems that fed-batch could be the acclimation method that will supply the required ammonia-tolerant bioaugmentation inocula in the future.

In conclusion, the outcome of this process provided mesophilic and thermophilic methanogenic communities that were capable to generate high methane yields despite high concentration of ammonia and been used, together with other cultures as bioaugmentation inocula in the following research activities of the project.

1.5.2 Bioaugmentation process development for continuous reactors

The main aim of the current research activity was to operate lab-scale CSTR reactors under high ammonia conditions testing the best ammonia tolerant methanogenic cultures as bioaugmentation inocula. Many experiments were performed during the MicrobStopNH3 project, which yielded 12 published and at least 6 under preparation ISI papers. The results of two of those experiments are presented in the current report that collectively cover all the main experimental challenges/parameters described in the proposal (e.g. mesophilic and thermophilic conditions, pure and mixed strains, different real-life substrates, etc.). Finally, based on the experimental findings, the basic microbiological and biochemical mechanisms that guarantee a successful bioaugmentation process are pinpointed.

1.5.2.1 Mesophilic bioaugmentation

In the first bioaugmentation study, hydrogenotrophic *Methanoculleus bourgensis* was bioaugmented in a mesophilic, ammonia-inhibited CSTR reactor fed mainly with microalgae, at extreme ammonia levels (i.e. 11 g NH_4^+-N L^{-1}). Immediately after bioaugmentation, methane production increased and reached 236 CH_4 g^{-1} VS establishing a new steady state (Figure 6). Compared to the baseline, 28% increase in methane yield was achieved after bioaugmentation, which demonstrated a successful strategy in recovering methane production from ammonia inhibited reactor. However, after the instability, the reactor entered a new steady state until the end of the experiment with an average production of 242 CH_4 g^{-1} VS, which was more than 31% higher than the baseline. Overall, after bioaugmentation, the reactor showed a significantly higher methane production efficiency compared to the inhibited steady state, and for the first time was demonstrated that bioaugmentation can alleviate ammonia inhibition of AD process fed with protein-rich, 3rd generation biomass.

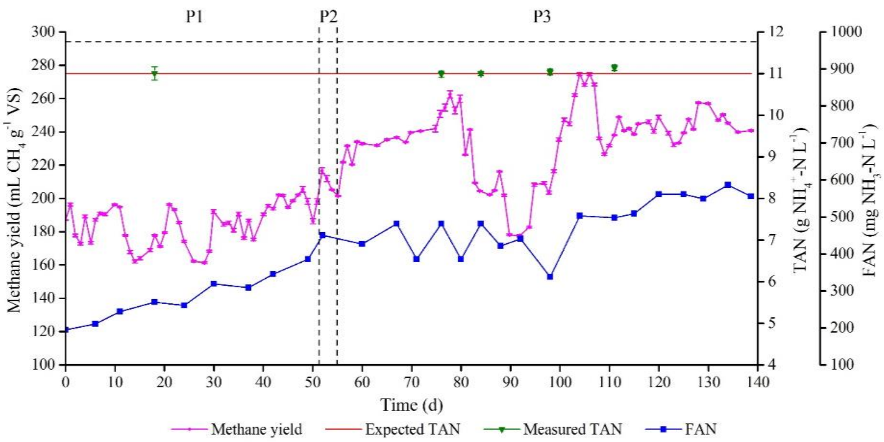

Figure 6. *Methane yield, TAN and FAN levels throughout the experimental period*

The VFA levels significantly decreased from more than 5 g L^{-1} to around 1 g L^{-1} after bioaugmentation (Figure 7), which coincided with the increase of methane yield discussed above. It seems that the decreased VFA levels due to bioaugmentation could mitigate the ammonia-VFA synergistic inhibition, thus resulted in a higher methane production. At the same time, the pH increased slightly after bioaugmentation, which might be due to the VFA consumption, but it remained within the optimal pH range (6.5-8.5) of AD process.

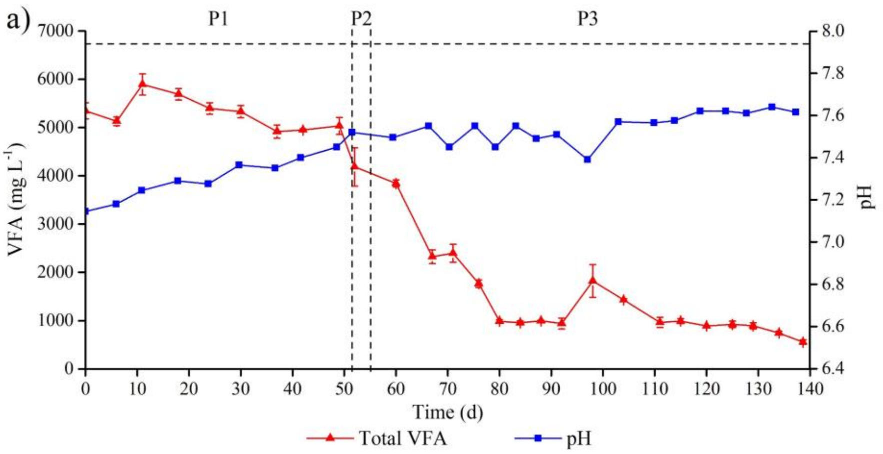

Figure 7. *VFA and pH variations of the CSTR reactor*

To identify the microbial dynamics, three samples were taken before the bioaugmentation, immediately after the bioaugmentation and at the end of the experiment, respectively. *Methanobrevibacter acididurans*, *Methanosarcina soligelidi* and *Methanoculleus bourgensis* were the three most abundant methanogens in all the samples (Figure 9). The bioaugmented *M. bourgensis* was found in all the samples, however, its relative abundance with respect to the archaea community increased from 3.6% before bioaugmentation to 6.7% after bioaugmentation. Although *M. bourgensis* was not the dominant methanogen, it seems that it was capable to drive the methanogenic microbiome towards a more efficient methanogenesis process. This has been described as a "microbiological domino effect", where the bioaugmented culture, even at a relative low abundance, is sufficient to drive the establishment of a new community. In fact, the bioaugmented *M. bourgensis* in this study triggered microbial community changes. The most possible explanation for the changes that *M. bourgensis* triggered was that reduced the hydrogen partial pressure by consuming hydrogen, which created thermodynamically favourable conditions for VFAs degradation, such as acetate and propionate due to their sensitivity to hydrogen. The reduced VFA levels further mitigated the synergistic inhibition of ammonia and VFA, and allowed the growth of other methanogens, such as aceticlastic *M. soligelidi*. As a result, immediately after bioaugmentation, *M. soligelidi* increased its relative abundance from 36.3% to 66.1% and reached 86.9% at the end of the experiment.

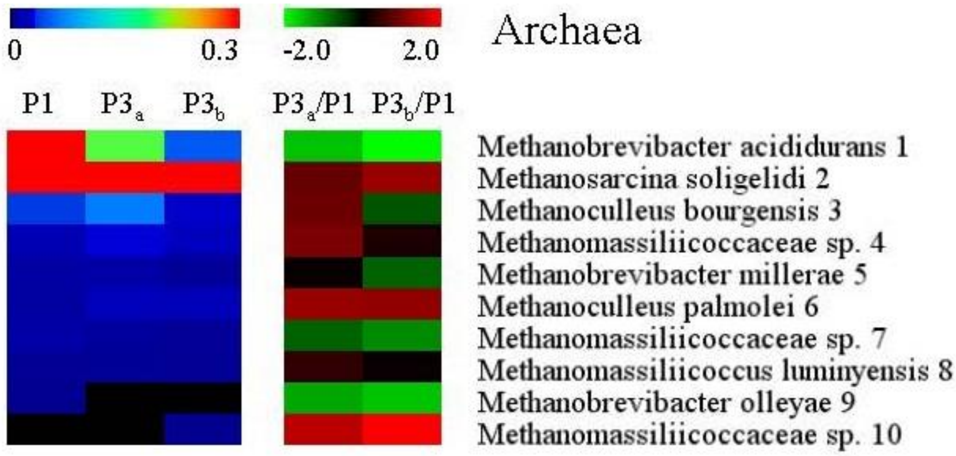

Figure 8. *Relative abundance (%) (left part) and the corresponding folds change (right part) for the interesting archaea at different phases.*

Overall, the "microbiological domino effect" triggered by the bioaugmented *M. bourgensis* was the most possible mechanism for the success of bioaugmentation in the current experimental study. This indicated that an efficient community could be established even with the less dominant bioaugmentation culture (Figure 9). Specifically, during the ammonia-induced inhibited steady state, both bacteria and archaea were inhibited primarily by the high hydrogen partial pressure and the ammonia-VFA synergistic inhibitory effect. However, the hydrogen partial pressure was reduced due to hydrogen consumption by the bioaugmented hydrogenotrophic *M. bourgensis*, thus resulted in VFAs degradation and synergistic inhibition mitigation. Therefore, overall favourable conditions were created for the growth of ammonia-tolerant bacteria and archaea. Finally, as a result of the aforementioned "microbiological domino effect", the whole microbial community was driven towards a more efficient community degrading protein-rich substrate, evidenced by the increased relative abundance of ammonia-tolerant methanogens.

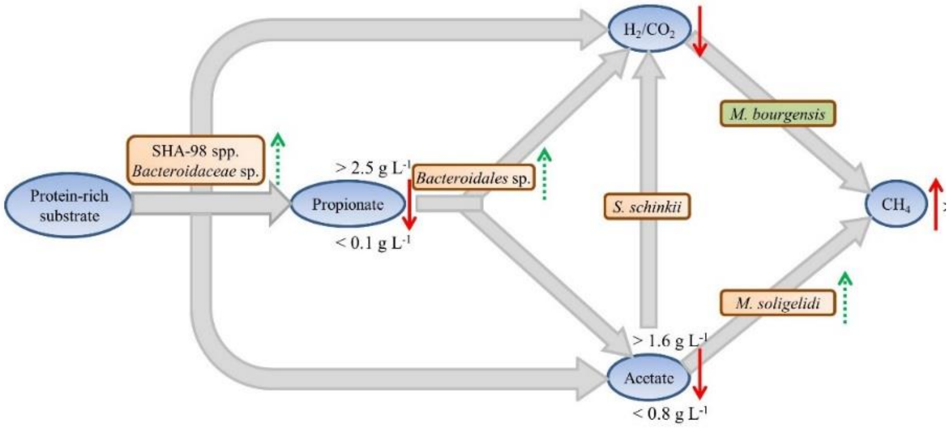

Figure 9. *The proposed working mechanism of the successful bioaugmentation of M. bourgensis. Red solid and green dashed arrows present the change of the compound concentration and the OUT's relative abundance, respectively.*

1.5.2.2 Thermophilic bioaugmentation

In the second bioaugmentation study, two bioaugmentation inocula (an enriched culture, and a mixed culture composed 50/50 by *Methanoculleus thermophilus* and the enriched culture) on the recovery of ammonia-inhibited (at 5 g NH_4^+-N L^{-1}) thermophilic continuous reactors was assessed. Three identical lab-scale CSTR reactors (R_{ctrl}, R_{enr} and R_{mix}) fed with cattle manure were used in the current study, operating under thermophilic (53±1°C) conditions with HRT of 15 days, and organic loading rate (OLR) of 2.50 g VS L^{-1} d^{-1} throughout the experiment.

Immediately after bioaugmentation, the methane production of R_{mix} (bioaugmentation with the mixed culture) increased by 17% compared to baseline inhibited production and established a steady state with an average yield of 130 mL CH_4 g^{-1} VS (78% recovery) until the end of the experiment (Figure 11a). At the same time R_{enr}, which was bioaugmented with only the enriched consortium, also increased its methane production with an average methane yield of 133 mL CH_4 g^{-1} VS (80% recovery). R_{ctrl} marginally increased its methane yield from 101 to 117 mL CH_4 g^{-1} VS at the last HRT of the experiment, indicating a degree of adaptation of the AD microbiome to the ammonia toxicity, which nevertheless it was still significantly lower compared to R_{mix} and R_{enr}. Thus, for the first time bioaugmentation was applied successfully to alleviate ammonia inhibition in thermophilic CSTR reactors since R_{enr} and R_{mix} improved the methane production efficiency by 11-13% compared to R_{ctrl}. Another interesting finding was that the methane production was recovered much faster in R_{mix} than R_{enr}, manifested by 15% higher overall methane production during the first ten days after bioaugmentation. This indicates that the mixed culture was a better bioaugmentation inoculum compared to the enriched culture, which demonstrated the importance of hydrogenotrophic methanogens included in the mixed culture. A possible explanation for this behaviour is that the instant hydrogen partial pressure removal by hydrogenotrophic *M. thermophilus* created better thermodynamic conditions for the overall AD process.

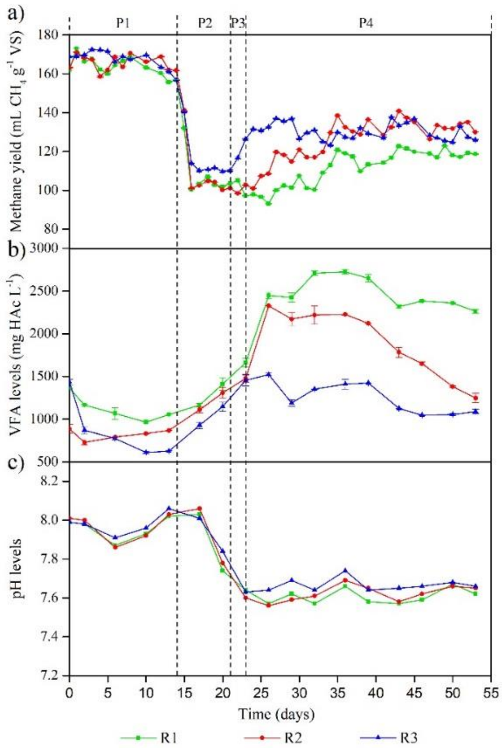

Figure 10. *The performance of the three reactors at different experimental phases: a) methane yield, b) total VFA levels and c) pH. P1, P2, P3, P4 stands for the four different experimental phases of the experiment, respectively.*

In general, the VFA accumulation of each reactor matched with the methane production variations. Specifically, before increasing the ammonia levels, the VFA levels of all the reactors were inside the normal threshold (<1500 mg L^{-1}) for CSTR reactors (Figure 11b). As expected, the VFA levels increased rapidly after increasing ammonia levels and exceeded the 1500 mg L^{-1} threshold. Moreover, The VFA increase also supports that methanogenesis was more susceptible to ammonia inhibition compared to acetogenesis. Specifically, the VFA levels in the R_{ctrl} stabilized between 2200 to 2700 mg L^{-} while the VFA levels in R_{mix} and R_{enr} decreased rapidly and returned below 1250 mg L^{-11} at the end of the experiment, demonstrating the immediate positive bioaugmentation effect. The pH levels fluctuated between 7.85 and 8.05 before the addition of ammonia, and decreased to lower levels varying between 7.55 and 7.75 due to the VFA accumulation after ammonia addition (Figure 11c). However, due to the high buffer capacity of manure the pH levels remained within the optimal pH range (6.5-8.5) for AD process.

To elucidate how the bioaugmentation cultures affected the microbial composition, four samples were taken from each reactor before TAN increase, after TAN increase but before bioaugmentation, immediately after bioaugmentation and at the end of the experiment, respectively. The most dominant methanogens were always *Methanothermobacter* sp.1 in all three reactors with relative abundance among the methanogens between 60 to 83% (Figure 12), verifying a well-documented strong hydrogenotrophic methanogenic activity in thermophilic reactors. Moreover, the relative abundance of *Methanothermobacter* sp.1 decreased significantly after the addition of ammonia, but it increased back during up to the initial levels for reactors R_{ctrl} and R_{enr}, indicating its ability to adapt at high ammonia levels. However, although *Methanothermobacter* sp. kept being dominant, its relative abundance in R_{mix} decreased significantly at the end, which could be explained by the increased abundance of bioaugmented *M. thermophilus*.

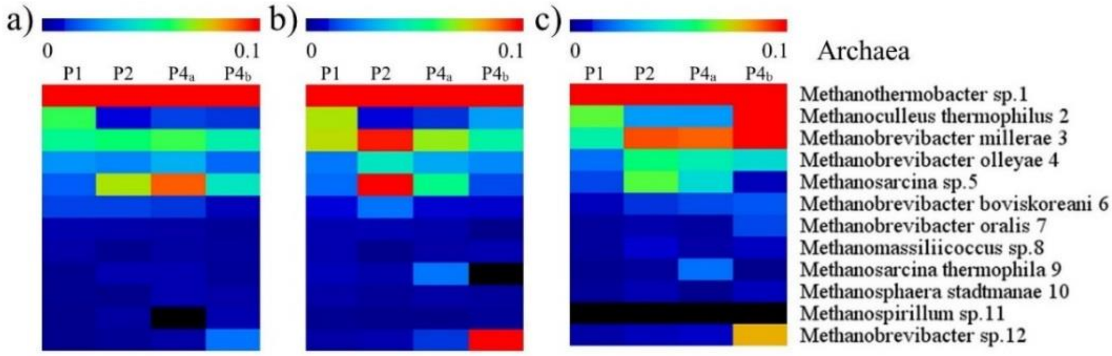

Figure 11. *Relative abundance shown as heat map for the interesting archaea at different phases in: a)* R_{ctrl}*, b)* R_{enr}*, c)* R_{mix}*,*

Overall, it seemed once again that the immediate reduction of the hydrogen partial pressure by the bioaugmented hydrogenotrophic methanogen was the main reason for a fast and more efficient bioaugmentation. Even though both bioaugmentation inocula resulted in an improved reactor performance, the mixed culture had a faster effect in recovering the methane production compared to the enriched culture, which indicated that the hydrogenotrophic *M. thermophilus* was the key to a successful bioaugmentation effect.

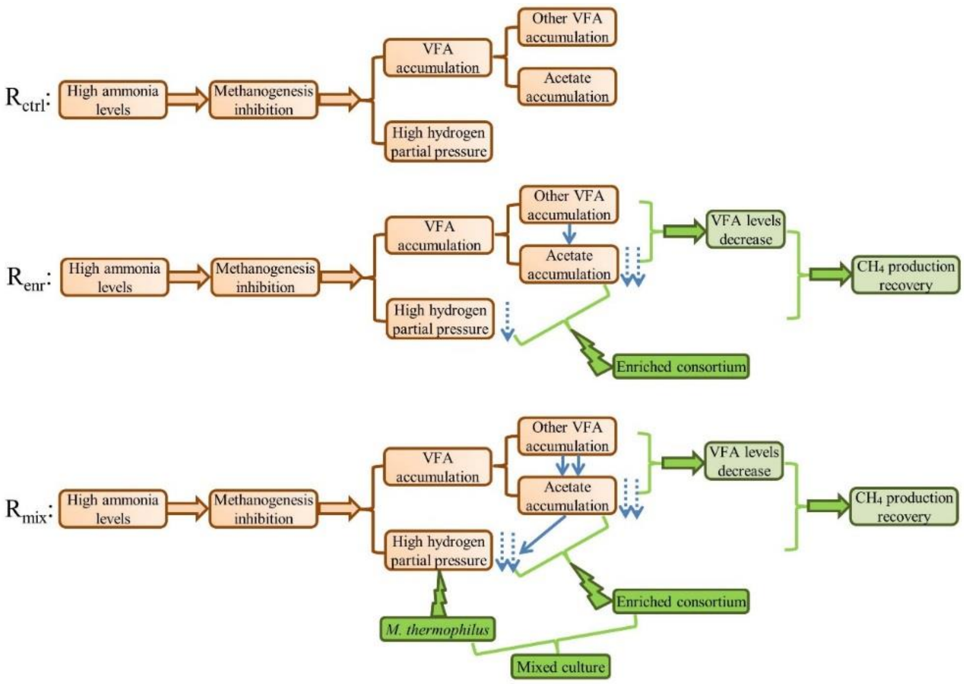

Figure 12. *Proposed mechanisms depicting the effects of bioaugmentation on the three reactors. The blue solid and dashed arrows present the conversion direction and decrease of the compounds' concentration, respectively, and two arrows stand for stronger effect compared to a single arrow. The orange and green processes stand for the reactors' performance before and after bioaugmentation, respectively.*

Based on the evaluation of all operational and microbiological data, a potential bioaugmentation working mechanism was proposed (Figure 12). Specifically, the instantly decreased hydrogen partial pressure, due to the consumption by *M. thermophilus* in R_{mix}, ceased homoacetogenesis, increased SAO and created favourable conditions (Eq. (1), (2), (4)) for VFA degradation by enhancing acetogenesis. Meanwhile, the enriched consortium (mainly aceticlastic *M. thermophila*) in the mixed culture also degraded acetate directly. Therefore, the VFA-ammonia synergistic inhibitory effect was alleviated rapidly due to the reduction of the hydrogen partial pressure, which resulted in a faster methane recovery compared to the other reactors.

When only the enriched culture was used (R_{enr}), it mainly increased acetate degradation, but it did not significantly contributed to the hydrogen partial pressure reduction. This resulted in a slower acetogenic step from other VFAs. Therefore, both strategies alleviated ammonia inhibition, but the bioaugmentation only with the enriched culture did not recover the methane production as fast as the bioaugmentation with the mixed culture.

In conclusion, the results demonstrated that the basic mechanism driving a successful and fast bioaugmentation process in ammonia inhibited CSTR reactors includes the instant hydrogen partial pressure reduction by bioaugmented ammonia-tolerant hydrogenotrophic methanogens that allows a thermodynamic syntrophic acetate oxidising process, which consequently decreases the VFA levels and thus, improves the overall AD process. Finally, it showed for the first time that bioaugmentation with ammonia-tolerant cultures can alleviate ammonia inhibition in thermophilic continuous reactors.

1.5.3 Pilot-scale verification

In WP3, the optimal bioaugmentation inoculum and bioaugmentation protocol, which are the outcomes of WP1 and WP2, were tested in a 500 L pilot biogas reactor using ammonia rich substrates (i.e. organic fraction of municipal solid waste-OFMSW).

1.5.3.1 Bioaugmentation inoculum

A number of 12 batch reactors with total working volume of ≈20 L and synthetically constructed media were used to produce the necessary bioaugmentation inoculum volume according to optimum bioaugmentation protocol determined in WP2. At the end of this task, a sufficient amount of ammonia tolerant inoculum (*Methanoculleus Bourgensis*) was produced, in order to subsequently be bioaugmented into the pilot-scale reactor.

Figure 13. *The bioaugmentation inoculum bioaugmented in the pilot-scale reactor.*

1.5.3.2 Pilot-scale bioaugmentation

The 500 L pilot-scale reactor fed with bio-pulped (mechanically pretreated) OFMSW at mesophilic conditions (37°C) was bioaugmented (Figure 14). The pilot-scale reactor had a hydraulic retention time (HRT) of 20 days and an organic loading rate (OLR) of 3.1±0.4 g VS $L^{-1}_{reactor}$ d^{-1}. The reactor operated for more than one HRT under a steady state before it was bioaugmented with 2% v/v the bioaugmentation inoculum. Bioaugmentation with the ammonia tolerant inoculum took place on day 21 of the experiment.

Figure 14. *The pilot-scale reactor set up and the bioaugmentation process.*

The results showed that immediately after bioaugmentation the methane production rate was increased by more than 40% compared to the methane production rate just before bioaugmentation (Figure 15). Additionally, after bioaugmentation the reactor recovered between 40 and 80% the previous uninhibited methane production (no inhibition period, Figure 15). The large range of this recovery can be attributed to the significant variations in the day to day production of a large-scale biogas reactor due to the changes in the homogeneity of the substrate. Nonetheless, the first ever pilot-scale bioaugmentation application seems to be very promising and paves the way for a future full-scale application of the MicrobStopNH_3 bioaugmentation technology.

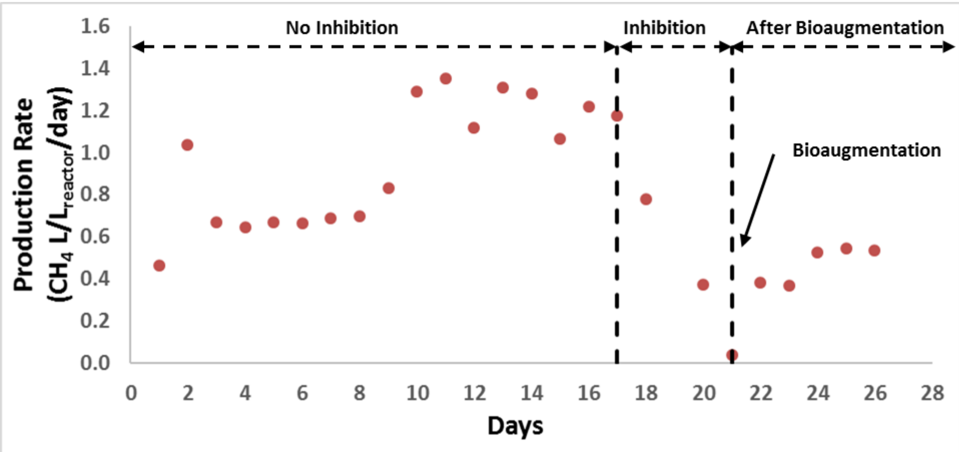

Figure 15. *Methane production of the pilot-scale reactor before and after bioaugmentation.*

1.5.4 System analysis

The main aim of the current research activity was to perform a technological study of the process that will include the analysis of potentials and technological constraints of all bioaugmentation parameters, as well as the scenario of applying bioaugmentation in commercial scale anaerobic reactors. Additionally, a specific environmental assessment to examine environmental impacts of the technology as well as preliminary economic impacts compared to a normal scenario of digesting ammonia-rich substrates was performed.

Therefore, an environmental and economic assessment of bioaugmentation as a strategy to overcome ammonia inhibition on anaerobic digestion processes was conducted (Figure 16). The option to employ this solution to mesophilic and thermophilic reactors was analysed in terms of its effect on climate change and economic incomes, based on its positive results in increase methane production registered in laboratory experiments. In order to understand the applicability of this technology at larger scales, besides the experimental results derived from the research activities of the MicrobStopNH_3 project, real life data for Denmark and Italy were used and compared.

Specifically, an environmental and cost assessment of bioaugmentation strategy as compared with a plant running at "inhibited steady state" was performed with the reference process defined in terms of process steps, material and energy flows involved, timeframe (2018) and country of application (i.e. Denmark and Italy).

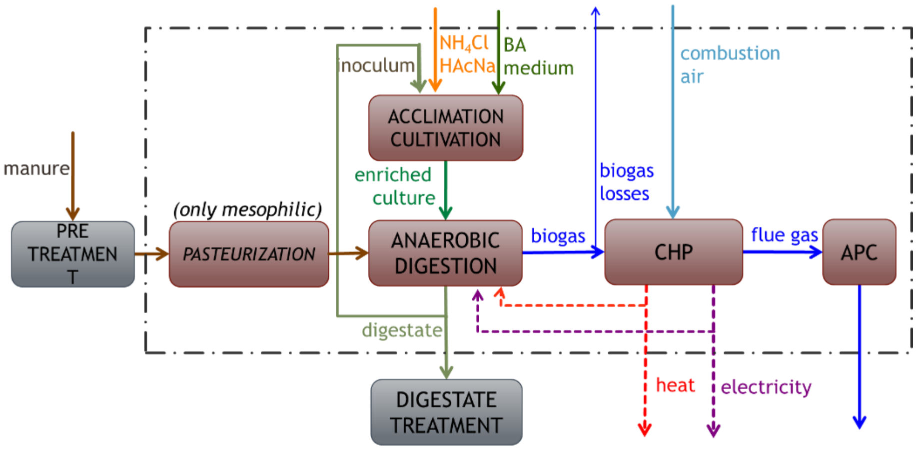

Figure 16. *Material flow scheme for bioaugmentation system analysis*

The economic analysis was performed on operational costs and revenues and it resulted in a greater income for the biogas plants when bioaugmentation was uses. The biogas plant operational temperature was also considered, with the mesophilic case being consistently more efficient than the thermophilic one, where ammonia inhibition had a stronger impact. Overall, in terms of environmental focus, climate change and emissions avoidance due to substituted energy production, the results showed a positive outcome using the bioaugmentation for both countries, which was more profound in the Italian case compared to Denmark. The specific results of the system analysis are presented in the following three subsections, and the functional unit of kg CO_2 equivalents per ton of wet waste entering the biogas plant was used.

1.5.4.1 Environmental performance

The overall results for the different scenarios applied to the two reference countries showed that the savings in terms of emissions are higher in the Italian case, where the substitute electricity and heat are mostly from fossil sources compared to Denmark where more alternative energy sources are used (Figure 17).

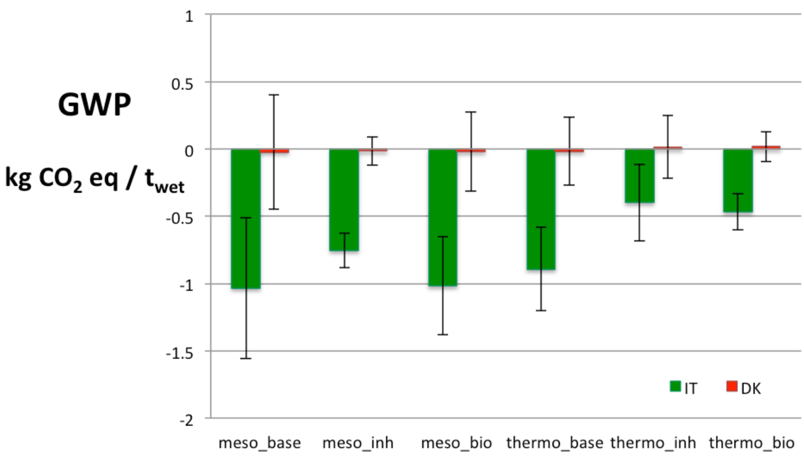

Figure 17. *Global Warming Potential results with standard deviation Denmark and Italy (meso: mesophilic conditions, thermo: thermophilic conditions, base: baseline case with no inhibition, inh: case in "inhibited steady state", bio: case with bioaugmentation).*

Both in the mesophilic and thermophilic operating conditions, bioaugmented biogas reactors have significantly lower environmental impact than the reactors operating under "inhibited steady state". This is a promising result, especially for Italy, highlighting how the bioaugmentation strategy can improve the overall plant efficiency and consequently the impact of anaerobic digestion on the environment. The effect of the reactor working temperature is also relevant. In fact, there is a more significant improvement from bioaugmentation in mesophilic compared to thermophilic conditions due to the influence of temperature on the dissociation equilibrium of FAN, and thus the higher inhibitory effect on the AD process.

The analysis on the relevant impact sources revealed that the emissions from biogas losses and the avoided impacts related to electricity and heat production were potential the primer contributors to the formulation of the global warming impact (Figure 18). Since these were clearly the main sources of impact, their determination could result in major errors. The differences between the two countries were carried in the substituted electricity and heat mix, which effected the contributions of avoided heat production, avoided electricity production, digester and pasteurization energy use. These were all higher in the Italian case, and the negative contributions from substituted energy made the total Italian global warming impact lower compared to the Danish one.

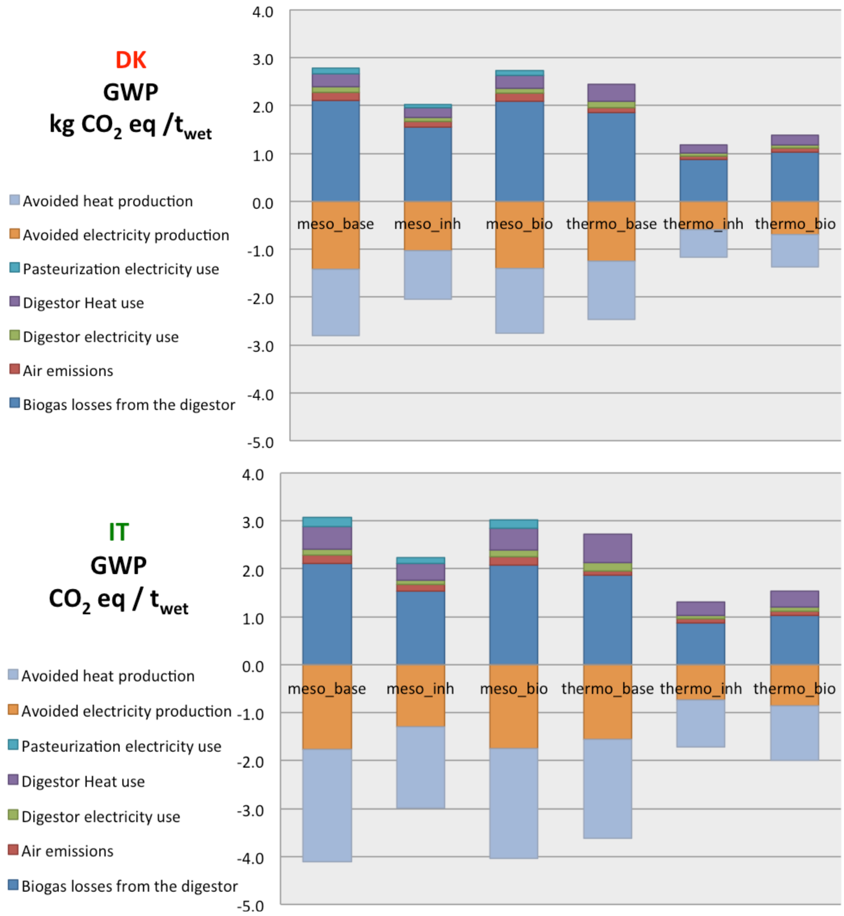

Figure 18. *Global Warming impact contributions for Denmark and Italy (meso: mesophilic conditions, thermo: thermophilic conditions, base: baseline case with no inhibition, inh: case in "inhibited steady state", bio: case with bioaugmentation).*

1.5.4.2 Economic feasibility

The economic assessment for the different scenarios applied to the two reference countries showed that the relative preference between the different scenarios within each country was the same as in the environmental assessment (Figure 19).

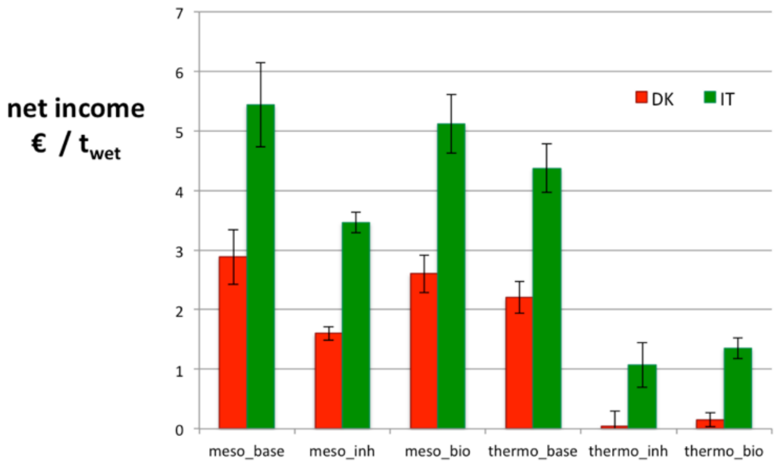

Figure 19. *Economic assessment results as net income for Denmark and Italy (meso: mesophilic conditions, thermo: thermophilic conditions, base: baseline case with no inhibition, inh: case in "inhibited steady state", bio: case with bioaugmentation).*

Specifically, bioaugmented reactors were yielding significantly higher revenues compared to ones that running under "inhibited steady state". Once again, the economic benefit in the Italian based scenarios were yielding better than the Danish ones. The final numbers followed the behaviour of methane production values. In fact the higher the methane production, the higher the energy produced, the higher the revenues but also the avoided impacts. Hence, it is possible to claim that the proposed bioaugmentation strategies would have positive effects on the environmental performance as well as the economic revenues of ammonia inhibited biogas reactors.

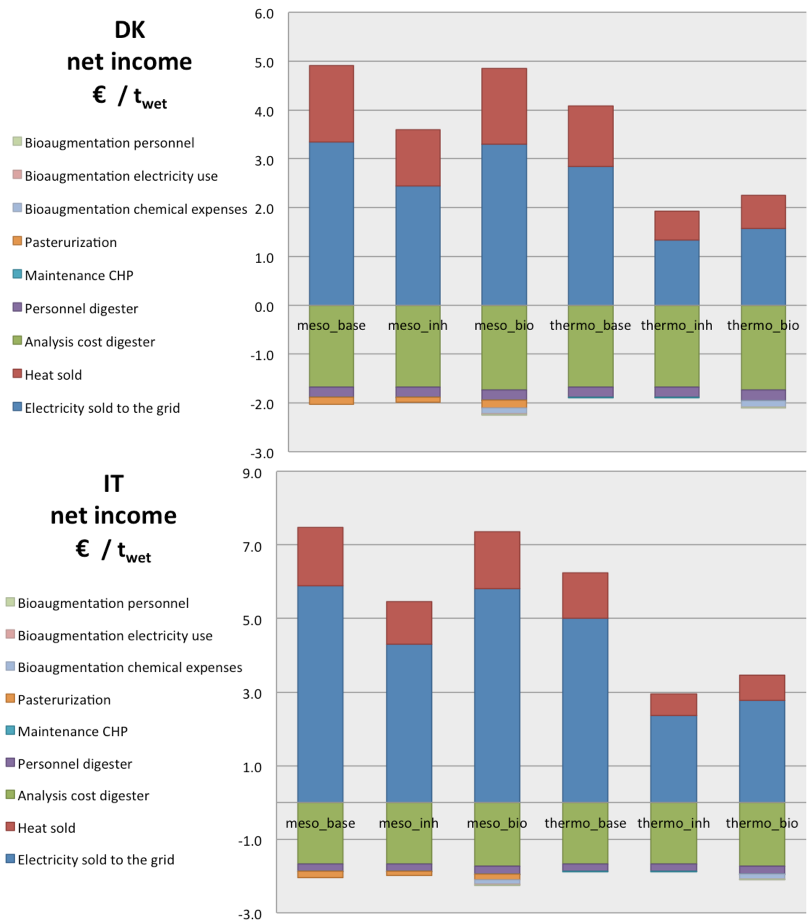

Figure 20. *Different contributions of the cost assessment for Denmark and Italy (meso: mesophilic conditions, thermo: thermophilic conditions, base: baseline case with no inhibition, inh: case in "inhibited steady state", bio: case with bioaugmentation).*

The analysed scenarios produced different results due to higher revenues in the cases of higher electrical and thermal energy production from the combustion of more biogas. The difference between the two countries lies on the price of electricity: since in Denmark the price is lower, the revenues were less than the Italian ones, and consequently so was the predicted net income (figure 20). Thus, it becomes clear that the electricity price is a decisive parameter.

1.5.5 The students of the MicrobStopNH$_3$

During the 50 months of the MicrobStopNH$_3$, at least 19 students have participated in the project activities. In the following paragraphs, their names and specific activities are presented. The specific student activity that corresponds to project milestones is indicated with the milestones abbreviations (M1-M4).

1.5.5.1 PhD Students

Three DTU PhD students have participated in the MicrobStopNH$_3$ project activities and two of them have already graduated.

- Miao Yan. "Powdered bioaugmentation inocula to alleviate ammonia toxicity in anaerobic digesters" (M1-M3).
- Hailin Tian. "Innovative bioaugmentation strategies to tackle ammonia inhibition in anaerobic digestion process" (M1-M3).
- Han Wang. "Innovative process for digesting high ammonia containing wastes" (M1, M2).

1.5.5.2 MSc thesis

Seven MSc student have conducted their MSc thesis as part of the MicrobStopNH$_3$ project activities and all of them graduated successfully. It has to be mentioned that the MSc thesis of Arnaud Jéglot received one of the six Veolia Performance Trophies 2018.

- Arnaud Jéglot. "Novel cultivation strategies of ammonia tolerant methanogenic consortia focused on: microbial growth, density and preservability" (M2, M3).
- Afroditi Panagiotou. "Bioaugmentation of ammonia tolerant methanogenic consortia in continuous reactors fed with ammonia-rich substrates" (M3).
- Konstantinos Kissas. "The impact of different ammonia sources on aceticlastic and hydrogenotrophic methanogenesis" (M2).
- Maria Ines Sobral Lupi Caetano. "Determination of the "critical biomass" for successful bioaugmentation strategies in ammonia-inhibited continuous anaerobic reactors" (M2, M3).
- Konstantinos Konstantopoulos. "Optimal cultivation processes and microbial characterization of ammonia-tolerant enriched methanogenic cultures" (M1, M2).
- Panagiotis Karachalios. "The significance of the ammonia–LCFA synergetic co-inhibition effect on the thermophilic biomethanation process" (M2).
- Enrico Mancini. "Acclimation processes of ammonia tolerant methanogenic consortia used as bioaugmentation inocula" (M1, M2).

1.5.5.3 Special MSc Courses

Nine special courses have been given as part of the MicrobStopNH$_3$ project activities.

- Estelle Maria Goonesekera, "Preliminary assessment of different biochar types to alleviate ammonia inhibition in anaerobic digestion" (M2, M3).
- Rosa Ferrigno, "Counteracting ammonia inhibition using bioaugmentation strategies of ammonia tolerant methanogenic inocula" (M2, M3).

- Dongdong Xie. "Combined alleviation of ammonia inhibition in anaerobic digestion by trace element addition and bioaugmentation of ammonia tolerant consortia"(M2, M3).
- Arnaud Tristan Arjuna Jeglot. "Optimal cultivation methods of high ammonia tolerant methanogenic consortia" (M2).
- Konstantinos Kissas. "Assessment of the effect of different ammonia sources on the aceticlastic and hydrogenotrophic methanogenesis" (M1, M2).
- Elizabeth Sembera. "Environmental and cost assessment of bioaugmentation techniques for anaerobic digestion" (M4).
- Maria Ines Sobral Lupi Caetand. "Preliminary assessment of the "critical biomass" of ammonia tolerant methanogenic consortia used as bioaugmentation" (M2, M3).
- Konstantinos Konstantopoulos. "Preliminary microbiological assessment of ammonia tolerant bioaugmentation inocula" (M1).
- Enrico Mancini. "Anaerobic digestion in protein-rich substrate with acclimatized inoculum" (M1).

1.6 Dissemination activities

The project participants have communicated the MicrobStopNH$_3$ results in dozens documented and undocumented dissemination activities including scientific manuscripts, posters, oral presentations, student lecture, keynote presentations etc. The documented project dissemination activities included 12 published ISI papers (first pages attached in the Annex), at least 6 ISI papers under preparation, at least 12 conference contributions. The specific dissemination activity that corresponds to project milestones is indicated with the milestones abbreviations (M1-M4).

1.6.1 ISI Papers published

- Tian, H., Yan, M., Treu, L., Angelidaki, I., Fotidis, I.A., (2019). "Hydrogenotrophic methanogens are the key for a successful bioaugmentation to alleviate ammonia inhibition in thermophilic anaerobic digesters." Bioresource technology, 122070 (M2, M3). https://doi.org/10.1016/j.biortech.2019.122070
- Tian, H., Mancini, E., Treu, L., Angelidaki, I., Fotidis, I.A., (2019). Bioaugmentation strategy for overcoming ammonia inhibition during biomethanation of a protein-rich substrate. Chemosphere 231, 415-422 (M2, M3). https://doi.org/10.1016/j.chemosphere.2019.05.140
- Yan, M., Fotidis, I.A., Tian, H., Khoshnevisan, B., Treu, L., Tsapekos, P., Angelidaki, I., (2019). Acclimatization contributes to stable anaerobic digestion of organic fraction of municipal solid waste under extreme ammonia levels: Focusing on microbial community dynamics. Bioresource Technology 286, 121376 (M1). https://doi.org/10.1016/j.biortech.2019.121376
- Tian, H., Treu, L., Konstantopoulos, K., Fotidis, I.A., Angelidaki, I., (2018). 16s rRNA gene sequencing and radioisotopic analysis reveal the composition of ammonia acclimatized methanogenic consortia. Bioresource Technology 272, 54-62 (M1). https://doi.org/10.1016/j.biortech.2018.09.128
- Tian, H., Karachalios, P., Angelidaki, I., Fotidis, I.A., (2018). A proposed mechanism for the ammonia-LCFA synergetic co-inhibition effect on anaerobic digestion process. Chemical Engineering Journal 349, 574-580 (M2). https://doi.org/10.1016/j.cej.2018.05.083
- Tian, H., Fotidis, I.A., Mancini, E., Treu, L., Mahdy, A., Ballesteros, M., González-Fernández, C., Angelidaki, I., (2018). Acclimation to extremely high ammonia levels in continuous biomethanation process and the associated microbial community dynamics. Bioresource Technology 247, 616-623 (M1). https://doi.org/10.1016/j.biortech.2017.09.148
- Tian, H., Fotidis, I.A., Kissas, K., Angelidaki, I., (2018). Effect of different ammonia sources on aceticlastic and hydrogenotrophic methanogens. Bioresource Technology 250, 390-397 (M2). https://doi.org/10.1016/j.biortech.2017.11.081
- Tian, H., Fotidis, I.A., Mancini, E., Angelidaki, I., (2017). Different cultivation methods to acclimatise ammonia-tolerant methanogenic consortia. Bioresource Technology 232, 1-9 (M1). https://doi.org/10.1016/j.biortech.2017.02.034
- Fotidis, I.A., Treu, L., Angelidaki, I. (2017). Enriched ammonia-tolerant methanogenic cultures as bioaugmentation inocula in continuous biomethanation processes. Journal of Cleaner Production 166, 1305-1313 (M1-M3). https://doi.org/10.1016/j.jclepro.2017.08.151Get rights and content
- Mahdy, A., Fotidis, I.A., Mancini, E., Ballesteros, M., González-Fernández, C., Angelidaki, I. (2017). Ammonia tolerant inocula provide a good base for anaerobic digestion of microalgae in 3rd generation biogas process. Bioresource Technology 225: 272-278 (M1-M3). https://doi.org/10.1016/j.biortech.2016.11.086

- Wang, H., Fotidis, I.A., and Angelidaki, I. (2016). Ammonia-LCFA synergetic co-inhibition effect in manure-based continuous biomethanation process. Bioresource Technology 209: 282-289 (M2). https://doi.org/10.1016/j.biortech.2016.03.003
- Wang, H., Fotidis, I.A., Angelidaki, I. (2015). Ammonia effect on hydrogenotrophic methanogens and syntrophic acetate oxidizing bacteria. FEMS Microbiology Ecology 91 (M2). https://doi.org/10.1093/femsec/fiv130

1.6.2 ISI papers under preparation

- Fotidis, I.A., Moretti, S., Damgaard, A., Astrup, T., Angelidaki, I. Environmental and cost assessment of bioaugmentation to alleviate ammonia inhibition on biomethanation processes (M4).
- Yan, M., Fotidis, I.A., Jéglot, A., Treu, L., Tian, H., Palomoa, A., Zhu, X., Angelidaki, I., Long term preservation and viability of ammonia tolerant methanogenic cultures (M1-M3).
- Yan, M., Campanaro, S., Treu, L., Tian, H., Zhu, X., Angelidaki, I., Fotidis, I.A. Insights into ammonia adaptations and methanogenic precursor oxidation by genome-guided analysis (M1-M3)
- Yan, M., Campanaro, S., Treu, L., Tian, H., Khoshnevisan, B., Tsapekos, P., Angelidaki, I., Fotidis, I.A. Bioaugmentation contributes to stable anaerobic digestion of organic fraction of municipal solid waste under extreme ammonia levels (M3).
- Yan, M., Jéglot, A., Angelidaki, I., Fotidis, I.A. Novel cultivation strategies of ammonia tolerant methanogenic consortia (M1).
- Yan, M., Caetano, M.I.S.L., Angelidaki, I., Fotidis, I.A. The critical biomass for successful bioaugmentation in ammonia-inhibited anaerobic reactors (M3).

1.6.3 Conferences, oral presentations and posters

- Ioannis Fotidis, Hailin Tian, Enrico Mancini, Laura Treu, Irini Angelidaki. Bioaugmentation alleviates ammonia inhibition during protein-rich substrate biomethanation. 16th World Congress of Anaerobic Digestion, Delft, June 23-27, 2019, poster, (M3).
- Miao Yan, Hailin Tian, Benyamin Khoshnevisan, Panagiotis Tsapekos, Irini Angelidaki, Ioannis Fotidis, Continuous biomethanation of organic fraction of municipal solid waste under extreme ammonia levels. 16th World Congress of Anaerobic Digestion, Delft, June 23-27, 2019, conference paper (M3).
- Miao Yan, Hailin Tian, Ioannis Fotidis, Benyamin Khoshnevisan, Panagiotis Tsapekos, Irini Angelidaki, Ammonia inhibition threshold during continuous biomethanation process. Sustain DTU conference-Creating Technology for a Sustainable Society, Kgs. Lyngby, Denmark, December 29-30, 2018, Abstract, poster and short oral presentation (M2).
- Hailin Tian, Ioannis Fotidis, Laura Treu, Ahmed Mahdy, Mercedes Ballesteros, Cristina González-Fernández, Irini Angelidaki,. Acclimation to extremely high ammonia levels during continuous biomethanation process. 15th World Congress of Anaerobic Digestion, Beijing, October 17-20, 2017, conference paper and oral presentation (M1).
- Hailin Tian, Ioannis Fotidis, Enrico Mancini, Irini Angelidaki. Combined inhibitory effect of ammonia and LCFA on biomethanation process. 15th World Congress of Anaerobic Digestion, Beijing, October 17-20, 2017, conference paper and oral presentation (M2).

- Ioannis Fotidis, Hailin Tian, Enrico Mancini, Irini Angelidaki. Batch, fed-batch and CSTR reactors as cultivation systems to acclimate ammonia tolerant methanogens. 15th World Congress of Anaerobic Digestion, Beijing, October 17-20, 2017, poster (M1).
- Hailin Tian, Ioannis A. Fotidis, Enrico Mancini, Irini Angelidaki. Microbial community dynamics during a successful acclimation process to extremely high ammonia levels in continuous anaerobic digester. Sustain DTU conference-Creating Technology for a Sustainable Society, Kgs. Lyngby, Denmark, December 6, 2017, abstract, poster and short oral presentation (M1, M2).
- Enrico Mancini, Hailin Tian, Ioannis A. Fotidis, Irini Angelidaki. Acclimation of ammonia tolerant methanogenic consortia using different bioreactor types. Sustain DTU conference-Creating Technology for a Sustainable Society, Kgs. Lyngby, Denmark, December 6, 2017, abstract, poster and short oral presentation (M1).
- Hailin Tian, Enrico Mancini, Ioannis Fotidis, Irini Angelidaki. Acclimation of continuous biomethanation process to extremely high ammonia levels. Biogas Science, Szeged, Hungary, 21-24 August 2016, abstract and oral presentation (M1).
- Ioannis Fotidis, Han Wan, Irini Angelidaki. Bioaugmentation of ammonia tolerant enriched methanogenic cultures: A microbiological process to efficiently digest ammonia-rich biomasses. 4th International Conference on Sustainable Solid Waste Management, Limassol, Cyprus June 23–25, 2016, abstract and oral presentation (M2, M3).
- Han Wan, Ioannis Fotidis, Irini Angelidaki. Effect of ammonia on hydrogenotrophic methanogens and syntrophic acetate oxidizing bacteria. Sustain DTU conference-Creating Technology for a Sustainable Society, Kgs. Lyngby, Denmark, December 17, 2015, abstract, poster and short oral presentation (M2).
- Ioannis Fotidis, Han Wan, Irini Angelidaki. Ammonia tolerant enriched methanogenic cultures as bioaugmentation inocula to alleviate ammonia inhibition in continuous anaerobic reactors. 14th World Congress of Anaerobic Digestion, Viña del Mar, Chile, November 15-18, 2015, conference paper, poster and oral presentation (M2, M3).

1.6.4 Utilization of project results

We believe that the results obtained in this project provide a good base for exploring the potential full scale application of the developed bioaugmentation technology. As it was mentioned before, the current project was focusing on technology development and pilot-scale application and not on commercialization of the process. Thus, there was no obligation for business plan development. With this in mind, we believe that the developed technology could be utilized commercially in the near future by the biogas plant operators due to the significant economic and environmental benefits that offers (based on the systems analysis 1.5.4).

Characteristically, animal manures (especially cow and pig) are a fundamental element for a healthy high rate methane production. However, manures have low methane yields and thus is necessary to co-digest them with other carbon-rich biomasses (up to 25% of co-substrate in Denmark), to increase productivity and render the process economical profitable. The amounts of the available co-substrates are limited and the need for new co-substrates with high methane yields growing exponentially the last years as the Danish government has set the goal of 50% use of all animal slurries in anaerobic reactors. This goal is very optimistic since the current use of animal slurries is estimated to be less than 20%.

MicrobStopNH$_3$ project developed and tested an innovative microbiological process that alleviates the ammonia inhibition effect in anaerobic reactors and thus allows the digestion of ammonia-rich wastes. These unique characteristics of MicrobStopNH_3 justify the necessity for a future, full-scale application of the developed technology. Since ammonia-rich substrates (e.g. food waste, slaughterhouse waste, mink and poultry manures, etc.) are abundant in Denmark and worldwide but, even if they have high methane potentials, are not widely used by the biogas plants today since they pose a thread for the stability and the efficiency of methane production. Additionally, it has been proven that many biogas plants are suffering from ammonia induced "inhibited steady-state" despite the fact that avoid using large amounts of ammonia-rich substrates. The conventional methods to tackle the ammonia toxicity problem (dilution, temperature lowering, etc.) are expensive, time consuming, reduce the reactor performance and don't provide a long-term solution to the problem. Thus, the potential use of the MicrobStopNH_3 bioaugmentation technology will allow these abundant but unwanted ammonia-rich biomasses to be used as co-substrates in the manure-based biogas reactors, enhancing their productivity and consequently their profitability.

We strongly believe that the MicrobStopNH_3 technology could counteract the ammonia toxicity effect on the anaerobic reactors efficiency, and thus ammonia-rich substrates could cover a large part of the need for additional co-digestion substrates that the Danish clean energy policies require. Even though the MicrobStopNH_3 project has shown great potentials for the bioaugmentation process by fulfilling all its objectives; there is still a lack of full-scale process approach and design for the commercial exploitation of the technology. This will have implications for the energy policy and future energy supply system in Denmark as well as in other countries. MicrobStopNH_3 technology can further increase the interest for investment in the biogas sector, can promote more construction activities in the biogas sector to fulfil the ambition of 100% fossil-free energy supply and reduced greenhouse gas emissions from 80% to 95%, by year 2050. The improved process efficiency and plant economy can also contribute to reduce the direct and indirect governmental subsidies. While, a successful biogas industry will allow Denmark to be less dependent on the natural gas imported from external, "unpredictable" suppliers.

As it has presented in section 1.5 (Project results and dissemination), three PhD students have participated in the project activities and many of the results of the project were part of their PhD thesis and the ISI papers that they have produced. During the project, some experimental activities were used during the 12136 Bioenergy technologies course (DTU) to train MSc and PhD students in ammonia inhibited anaerobic digestion in lab-scale activities. It is estimated that more than 40 students have been part of these activities between 2015 and 2019. Finally, at this stage the MicrobStopNH_3 partners do not consider applying for any patents.

1.7 Project conclusion and perspective

The MicrobStopNH$_3$ project proposed bioaugmentation as a novel technical approach to establish ammonia tolerant consortia in continuous digesters operating under high ammonia levels. The major conclusion of the project is that: it is technically possible to use bioaugmentation of ammonia tolerant methanogenic consortia to improve significantly (up to 40%) methane production of continuous anaerobic reactors suffering from ammonia toxicity.

During the project many lab-scale experiments and assessments were performed, thus there are many significant scientific conclusions drawn from them that have or will be included in the ISI publications of the project. With that in mind the, the main conclusions of the project are:

- Using the case-appropriate acclimatization strategy it is possible to create ammonia tolerant methanogenic consortia in batch, fed-batch and continuous reactors.
- Mixed mesophilic and thermophilic ammonia tolerant methanogenic consortia can be used as bioaugmentation inocula with similar or better efficiency compared to pure methanogenic cultures.
- The "critical biomass" (minimum amount) of bioaugmentation inoculum necessary for a successful bioaugmentation in continuous reactors is at least 2% v/v.
- The hydrogenotrophic methanogens are the keystone microbes for the success of the bioaugmentation process under extreme ammonia conditions, since they immediately reduce the hydrogen partial pressure, which affects positively the efficiency of many other catabolic processes.
- The pilot-scale verification of the developed bioaugmentation technology showed that the improvement in methane production is similar to the one achieved in the lab-scale continuous reactors' experiments.
- The developed bioaugmentation technology, if applied in full scale anaerobic reactors, could have a clearly positive effect on the climate change and emissions avoidance as well as a significant economic benefits for the biogas plants

We strongly believe that the technology developed in the MicrobStopNH$_3$ project can be utilised in full-scale reactors. In order to achieve this, the technology readiness level has to be moved pass the pilot-scale verification stage, the stakeholders in collaboration with the researchers have to invest funds and time in order to create a commercially applicable process that meets the requirements and the expectations of the industry. This commercially available bioaugmentation technology is the next major goal of the researchers involved in the MicrobStopNH$_3$ project as well as other researchers around the world that they have recently included bioaugmentation in their research activities, inspired by the MicrobStopNH$_3$ project results.

Annex

The first pages of the 12 Published ISI papers

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Bioresource Technology

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Hydrogenotrophic methanogens are the key for a successful bioaugmentation to alleviate ammonia inhibition in thermophilic anaerobic digesters

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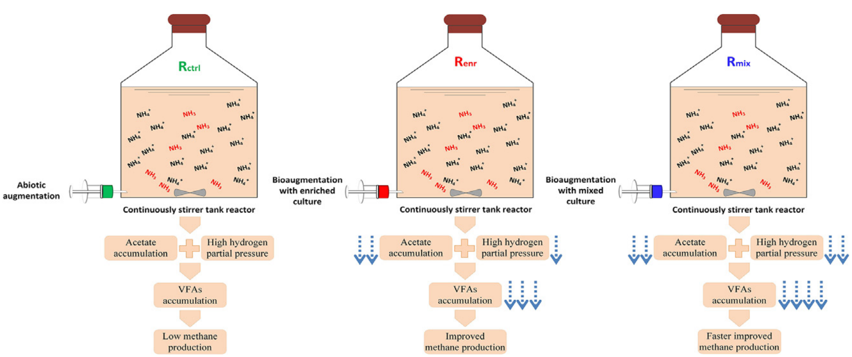
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Biogas
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Methanoculleus thermophilus
Methanosarcina thermophila

ABSTRACT

Bioaugmentation to alleviate ammonia inhibition under thermophilic anaerobic digestion has never been reported, as well as the working mechanism that allows a fast and successful bioaugmentation. Thus two bioaugmentation inocula (an enriched culture, and a mixed culture composed 50/50 by *Methanoculleus thermophilus* and the enriched culture) on the recovery of ammonia-inhibited thermophilic continuous reactors was assessed. The results showed that bioaugmentation improved methane yield by 11–13% and decreased the volatile fatty acids (VFA) by 45–52% compared to the control reactor (abiotic augmentation). Moreover, the importance of hydrogenotrophic methanogens to a fast and successful bioaugmentation was recognized. Specifically, the instant hydrogen partial pressure reduction by the bioaugmented hydrogenotroph created thermodynamically favourable conditions for the acetate oxidation process and consequently, the catabolism of other VFA. High-throughput sequencing results strengthened this explanation by showing that the bioaugmented *M. thermophilus* stimulated the growth of syntrophic acetate oxidising bacterium *Thermacetogenium phaeum*, immediately after bioaugmentation.

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Bioaugmentation strategy for overcoming ammonia inhibition during biomethanation of a protein-rich substrate

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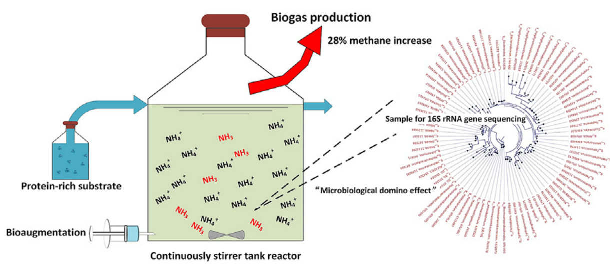
HIGHLIGHTS

- Bioaugmentation alleviated ammonia inhibition of a reactor fed with microalgae.
- Bioaugmented *M. bourgensis* increased methane yield by 28% and decreased VFA by 80%.
- Reduction of hydrogen pressure and propionate is crucial in alleviating inhibition.
- The bioaugmentation success lies on the triggered “microbiological domino effect”.
- *M. soligelidi* was the dominant methanogen three HRTs after bioaugmentation.

GRAPHICAL ABSTRACT



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Methanoculleus bourgensis
Hydrogen partial pressure

ABSTRACT

High ammonia levels inhibit anaerobic digestion (AD) process and bioaugmentation with ammonia tolerant methanogenic culture is proposed to alleviate ammonia inhibition. In the current study, hydrogenotrophic *Methanoculleus bourgensis* was bioaugmented in an ammonia-inhibited continuous reactor fed mainly with microalgae (a protein-rich biomass), at extreme ammonia levels (i.e. 11 g NH_4^+-N L^{-1}). The results showed 28% increase in methane production immediately after bioaugmentation. Moreover, volatile fatty acids decreased rapidly from more than 5 g L^{-1} to around 1 g L^{-1}, with a fast reduction in propionate concentration. High throughput 16s rRNA gene sequencing demonstrated that the bioaugmented *M. bourgensis* doubled its relative abundance after bioaugmentation. “Microbiological domino effect”, triggered by the bioaugmented *M. bourgensis* establishing a newly efficient community, was proposed as the working mechanism of the successful bioaugmentation. Additionally, a strong aceticlastic methanogenesis was found at the end of the experiment evidenced by the dominant presence of *Methanosarcina soligelidi* and the low abundance of syntrophic acetate oxidising bacteria at the final period. Overall, for the first time, this study proved the positive effect of bioaugmentation on ammonia inhibition alleviation of the microalgae-dominating fed reactor, paving the way of efficient utilization of other protein-rich substrates in the future.

1. Introduction

Biogas from anaerobic digestion (AD) is a renewable energy carrier, which plays an important role in substituting fossil fuel and

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Acclimatization contributes to stable anaerobic digestion of organic fraction of municipal solid waste under extreme ammonia levels: Focusing on microbial community dynamics

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ARTICLE INFO

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Ammonia acclimatization
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Methanosarcina soligelidi

ABSTRACT

The organic fraction of municipal solid waste (OFMSW) is an abundant and sustainable substrate for the anaerobic digestion (AD) process, yet ammonia released during OFMSW hydrolysis could result in suboptimal biogas production. Acclimatized ammonia tolerant microorganisms offer an efficient way to alleviate ammonia inhibition during AD. This study aimed to achieve an efficient AD of OFMSW under extreme ammonia levels and elucidate the dynamics of the acclimatized microbial community. Thus, two mesophilic continuous stirred tank reactors (CSTR), fed only with OFMSW, were successfully acclimatized up to 8.5 g NH_4^+-N/L, and their methane yields fluctuated < 10%, compared to the methane yields without ammonia addition. Microbiological analyses showed that *Methanosaeta concilii* and *Methanosarcina soligelidi* were the dominant methanogens at low and high ammonia levels, respectively. Whilst, a unique metabolic pathway shift, from aceticlastic to hydrogenotrophic methanogenesis, of *M. soligelidi* was identified during the acclimatization process.

1. Introduction

The increasing global need for natural resources and consumer goods leads to vast amounts of municipal solid waste (MSW). In 2018, more than 2.5 Mt of MSW were generated in the European Union (Eurostat, 2018), with 46% of them been organic (OFMSW: organic fraction of MSW) (Hoornweg and Bhada-Tata, 2012). Improper treatment methods of OFMSW can cause environmental and health issues (Fisher, 2006). Currently, most of the MSW is treated in one of the four ways: landfilling (24%), incineration (28%), recycling (29%), and composting (16%) (Eurostat, 2018). Landfilling and incineration methods do not take advantage of the organic faction and the nutrients of OFMSW and can lead to extra greenhouse gas emissions (Fisher, 2006; Eurostat, 2018). At the same time, huge consumption and shortage of fossil fuels motivate researchers to find alternative energy sources. Thus, new treatment technologies must be developed to not only offset the use of fossil fuels but also maximize the reuse of the vast amounts of the OFMSW and thereby maintain and recycle the useful nutrient back to agriculture.

Anaerobic digestion (AD) is a process that can convert organic waste into sustainable energy (biogas), via a series of interrelated microbial metabolisms (Campanaro et al., 2018). The AD process is divided in four steps (i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis), which are mediated primarily by bacteria and archaea (Angelidaki et al., 2011). In addition, the liquid product of the AD process (digestate) contains high levels of nitrogen and phosphorus, which can be potentially reused as biofertilizer or after extraction, as supplement for fermentation processes. In spite of these benefits, the degradation of OFMSW produces ammonia, a by-product from the catabolism of proteins, which can be toxic to the AD process, resulting in poor operational stability and reduced methane production efficiency (Tian et al., 2018b).

Total ammonia (TAN) is the sum of ammonium ions (NH_4^+) and free ammonia (FAN, NH_3), while FAN exists in an equilibrium defined by the pH and the temperature (Tian et al., 2017). As Koster and Lettinga (1988) reported, when ammonia concentrations ranged from 4 to 5.7 g NH_4^+-N/L, more than 56% of the methanogenic activity was inhibited in a granular sludge reactor. The reason is that FAN is freely membrane-permeable and hence, decreases methanogenic activity by interfering with the natural intracellular biological pathways of the methanogens (e.g. inhibiting specific methane synthesizing enzyme reaction) (Sprott and Patel, 1986). It is generally accepted that

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16s rRNA gene sequencing and radioisotopic analysis reveal the composition of ammonia acclimatized methanogenic consortia

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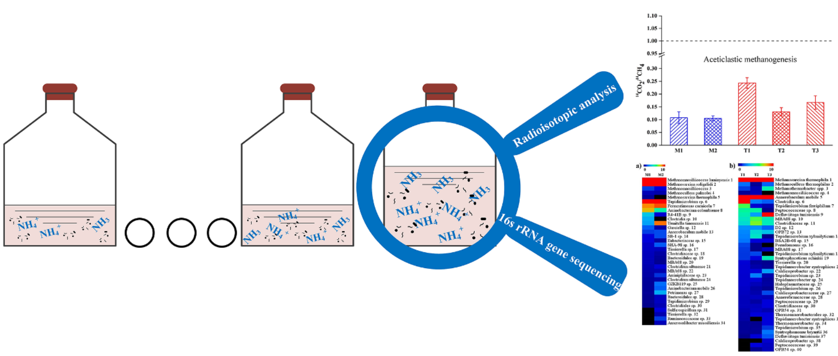
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Anaerobic digestion
Enriched culture
Methanogenic pathway
Ammonia-tolerant microbial community
Methanomassiliicoccus luminyensis

ABSTRACT

Different mesophilic and thermophilic methanogenic consortia were acclimatised and enriched to extreme total ammonia (9.0 and 5.0 g NH_4^+-N L^{-1}, respectively) and free ammonia (1.0 and 1.4 g NH_3-N L^{-1}, respectively) levels in this study. [2-^{14}C] acetate radioisotopic analyses showed the dominance of aceticlastic methanogenesis in all enriched consortia. According to 16S rRNA gene sequencing result, in mesophilic consortia, methylotrophic *Methanomassiliicoccus luminyensis* was predominant, followed by aceticlastic *Methanosarcina soligelidi*. A possible scenario explaining the dominance of *M. luminyensis* includes the use of methylamine produced by *Tissierella* spp. and biomass build-up by metabolizing acetate. Nevertheless, further studies are needed to pinpoint the exact metabolic pathway of *M. luminyensis*. In thermophilic consortia, aceticlastic *Methanosarcina thermophila* was the sole dominant methanogen. Overall, results derived from this study demonstrated the efficient biomethanation ability of these ammonia-tolerant methanogenic consortia, indicating a potential application of these consortia to solve ammonia toxicity problems in future full-scale reactors.

1. Introduction

Anaerobic digestion (AD) is a widely used biotechnological process to treat a variety of biowaste and recover renewable energy (CH_4). It is a complex biological process that is mediated by different microbes (bacteria and archaea) and consists of four steps, named hydrolysis, acidogenesis, acetogenesis and methanogenesis. Acetate is the most important precursor of methane, with two main pathways involved in its catabolism: aceticlastic methanogenesis (Eq. (1)) and syntrophic acetate oxidation (SAO) coupled with hydrogenotrophic

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Effect of different ammonia sources on aceticlastic and hydrogenotrophic methanogens

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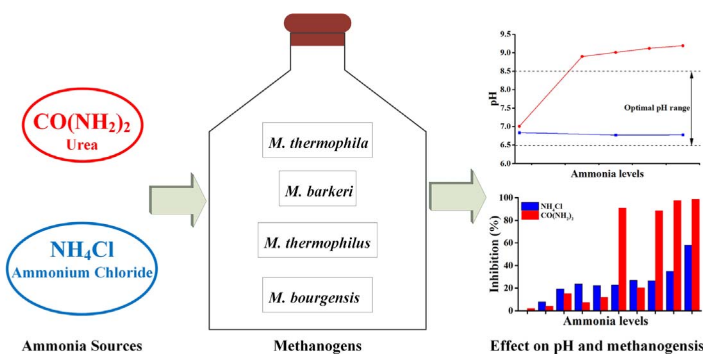
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Ammonia inhibition
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Anaerobic digestion
Pure strain
Urea

ABSTRACT

Ammonium chloride (NH_4Cl) was usually used as a model ammonia source to simulate ammonia inhibition during anaerobic digestion (AD) of nitrogen-rich feedstocks. However, ammonia in AD originates mainly from degradation of proteins, urea and nucleic acids, which is distinct from NH_4Cl. Thus, in this study, the inhibitory effect of a "natural" ammonia source (urea) and NH_4Cl, on four pure methanogenic strains (aceticlastic: *Methanosarcina thermophila*, *Methanosarcina barkeri*; hydrogenotrophic: *Methanoculleus bourgensis*, *Methanoculleus thermophilus*), was assessed under mesophilic (37 °C) and thermophilic (55 °C) conditions. The results showed that urea hydrolysis increased pH significantly to unsuitable levels for methanogenic growth, while NH_4Cl had a negligible effect on pH. After adjusting initial pH to 7 and 8, urea was significantly stronger inhibitor with longer lag phases to methanogenesis compared to NH_4Cl. Overall, urea seems to be more toxic on both aceticlastic and hydrogenotrophic methanogens compared to NH_4Cl under the same total and free ammonia levels.

1. Introduction

Biogas (a mixture of CH_4 and CO_2) is an attractive renewable energy (Holm-Nielsen et al., 2009), which is formed during anaerobic digestion (AD) of different biomasses. As one of the most promising and widely used green technologies, AD is a complex biological process with different microorganisms involved, which can reduce the waste pollution and offset part of the energy usage (Chynoweth et al., 2001). However, it is reported that some potential substrates are toxic to AD process by inhibiting the microorganisms' activity (Chen et al., 2008). Among these substrates, nitrogen-rich substrates stand out, due to the ammonia formation during their degradation. A low ammonia concentration ($< 200\ mg\ NH_4^+$-$N\ L^{-1}$) is beneficial to AD process; nevertheless, relatively high ammonia levels ($> 2000\ mg\ NH_4^+$-$N\ L^{-1}$) would inhibit

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Acclimation to extremely high ammonia levels in continuous biomethanation process and the associated microbial community dynamics

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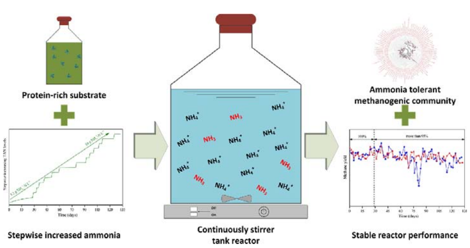
GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:
Methane
Ammonia inhibition
Microbial community
Syntrophic acetate oxidizer
Methanosarcina

ABSTRACT

Acclimatized anaerobic communities to high ammonia levels can offer a solution to the ammonia toxicity problem in biogas reactors. In the current study, a stepwise acclimation strategy up to 10 g NH_4^+-N L^{-1}, was performed in mesophilic (37 ± 1 °C) continuously stirred tank reactors. The reactors were co-digesting (20/80 based on volatile solid) cattle slurry and microalgae, a protein-rich, 3rd generation biomass. Throughout the acclimation period, methane production was stable with more than 95% of the uninhibited yield. Next generation 16S rRNA gene sequencing revealed a dramatic microbiome change throughout the ammonia acclimation process. *Clostridium ultunense*, a syntrophic acetate oxidizing bacteria, increased significantly alongside with hydrogenotrophic methanogen *Methanoculleus* spp., indicating strong hydrogenotrophic methanogenic activity at extreme ammonia levels (> 7 g NH_4^+-N L^{-1}). Overall, this study demonstrated for the first time that acclimation of methanogenic communities to extreme ammonia levels in continuous AD process is possible, by developing a specialised acclimation AD microbiome.

1. Introduction

Anaerobic digestion (AD) is a sustainable technology that can produce biogas and nutrient-rich bio-fertilizer from a broad variety of residual biomass (e.g. agricultural waste, food waste, and sewage sludge) (Karim et al., 2005). AD is a complex biological process, which comprises four main steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis, with a variety of microorganisms mediating each step. Acetate is the main precursor of methane production which follows two major methanogenic pathways: a) aceticlastic pathway and b) hydrogenotrophic pathway (syntrophic acetate oxidation (SAO) coupled with hydrogenotrophic methanogenesis). Aceticlastic pathway is mediated

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A proposed mechanism for the ammonia-LCFA synergetic co-inhibition effect on anaerobic digestion process

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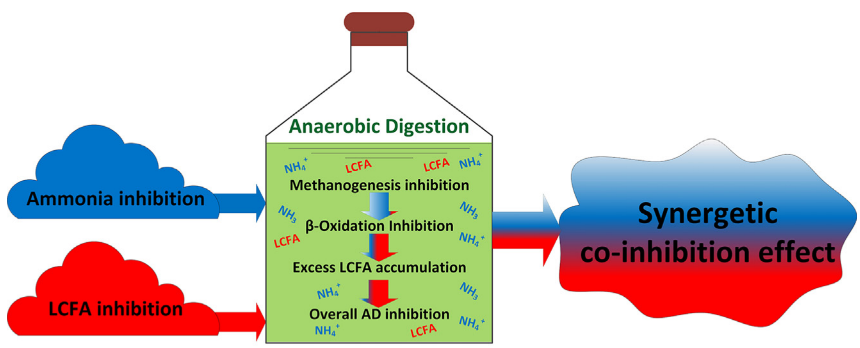
HIGHLIGHTS

- Identification of NH_3-LCFA synergetic effect in batch and continuous reactors.
- A new mechanism was proposed to explain the NH_3-LCFA synergetic effect.
- Excess LCFA levels due to β-oxidation inhibition by NH_3 trigger the synergism.
- Adaptation of the microbiome to the synergism is possible in continuous reactor.
- Different NH_3-LCFA levels cause the synergism in batch and continuous reactors.

GRAPHICAL ABSTRACT



ARTICLE INFO

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Anaerobic microbiome
Batch reactors
CSTR reactors
Methane
Toxicity

ABSTRACT

Ammonia and long chain fatty acids (LCFA) are two major inhibitors of the anaerobic digestion (AD) process. The individual inhibitory effect of each of these two inhibitors is well established; however, the combined co-inhibition effect has not been thoroughly assessed yet. In the current study, the ammonia-LCFA synergetic co-inhibition effect was investigated in both batch and continuous experiments. In the batch experiments, a clear ammonia-LCFA synergetic co-inhibitory effect was identified when the LCFA concentrations were higher than 0.05 g oleate L^{-1} and ammonia levels between 4.0 and 7.0 NH_4^+-N L^{-1}. This synergetic effect for LCFA and ammonia levels above 1.1 g oleate L^{-1} and 4.5 NH_4^+-N L^{-1}, respectively, was validated in continuous reactors experiments. Nevertheless, adaptation of the AD microbiome to this synergetic co-inhibition could occur after a period of continuous operation. A potential mechanism to explain the synergetic co-inhibition lies on the initial inhibition of methanogens caused by ammonia resulting in increased VFA and hydrogen concentrations, which in turn renders β-oxidation of LCFA thermodynamically unfavourable and thereby brings about further excess accumulation of LCFA and consequently higher unspecific toxicity of all AD steps. This is a vicious cycle, which makes the combined inhibition of the two toxicants more severe, compared to the sum of their individual inhibition effects at the same operational conditions.

1. Introduction

Anaerobic digestion (AD) is a widely used sustainable technology for bioenergy (CH_4) recovery from a variety of biowastes and wastewaters, such as industrial wastewater, agricultural and forestry residues, municipal sewage sludge etc. [1,2]. It is a complex biological process, consisting of four steps, i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis, which are mediated by different groups

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Enriched ammonia-tolerant methanogenic cultures as bioaugmentation inocula in continuous biomethanation processes

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Hydrogenotrophic methanogen

ABSTRACT

Many ammonia-rich biomass sources, such as manures and protein-rich substrates, are potential inhibitors of the anaerobic digestion (AD) process. It was previously demonstrated that bioaugmentation of *Methanoculleus bourgensis* MS2^T in an ammonia inhibited process in a continuous stirred tank reactor (CSTR), resulted in up to 90% recovery of the methane production compared to the uninhibited production. However, cultivation of pure strains has practical difficulties due to the need of special growth media and sterile conditions. In contrast, acclimatized enriched cultures have minor sterility requirements. In the current study, an enriched ammonia-tolerant methanogenic culture was bioaugmented in a CSTR reactor operating under ammonia-induced, inhibited-steady-state. The results demonstrated that bioaugmentation, completely counteracted the ammonia toxicity effect. This indicates that a commercial application of bioaugmentation could improve up to 36% the methane production, the greenhouse gas reduction efficiency and the gross revenue of ammonia inhibited full scale biogas reactors. 16S rRNA gene sequencing showed that bioaugmentation changed the microbial composition of the reactors resulting in higher bacterial and lower archaeal community diversity. The bioaugmented reactor showed a fourfold increase of the abundance of the bioaugmented methanogens compared to the control reactor. This indicates that ammonia-tolerant methanogens established well in the ammonia-inhibited reactor and dominated over the domestic methanogenic population. Finally, this study showed that the enriched culture alleviated ammonia toxicity 25% more efficiently than the previously used pure culture.

1. Introduction

Vast amounts of ammonia-rich organic wastes are produced yearly from the agricultural and the food industrial sectors (Kovács et al., 2013). Anaerobic digestion (AD) is one of the most effective methods to treat this waste, as it provides energy (methane) and a bio-fertilizer (digestate) (Tampio et al., 2016). Moreover, some manures (e.g. pig, poultry etc.) that are often used as substrates in biogas reactors contain high amounts of urea. Ammonia is a well-known inhibitor of the AD process (Westerholm et al., 2015). It has been widely shown that free ammonia (unionised, NH_3) is the most toxic form of the total ammonia (NH_4^++NH_3) for the methanogenic communities mediating the AD process (Rajagopal et al., 2013). The NH_3 levels depend on the total ammonia concentration in a reactor and on the NH_4^+/NH_3 equilibrium, which is affected by the temperature and the pH (Yenigün and Demirel, 2013).

Many solutions have been proposed to solve the ammonia inhibition problem. Up to now, the two most common methods are lowering the operating temperature and diluting the reactor content with water (Kelleher et al., 2002; Nielsen and Angelidaki, 2008). Nevertheless, these methods can counteract the ammonia toxicity only to a limited extend, are uneconomical and do not provide a permanent solution (Massé et al., 2014).

Europe has currently more than 17,240 biogas plants and most of them use combined heat and power (CHP) units to generate electricity on site, with an average efficiency of 40% (European Biogas Association, 2015; Herbes et al., 2016). At the same time, it has been found that several of the European biogas reactors are seriously affected by ammonia toxicity, leaving unexploited more than 30% of their methane potential, while operating in an ammonia induced inhibited-steady-state (Duan et al., 2012; Fotidis et al., 2014a). This suboptimal, but apparently stable process is resulting in severe operational problems with increased CO_2

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Different cultivation methods to acclimatise ammonia-tolerant methanogenic consortia

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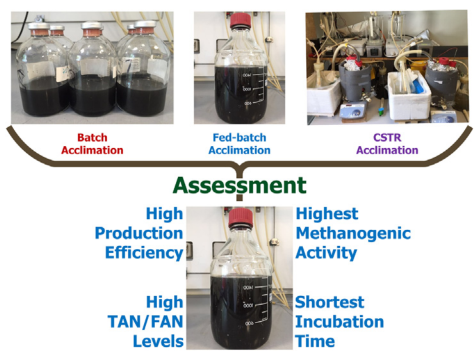
HIGHLIGHTS

- Fed-batch was the most efficient method to acclimatise ammonia tolerant consortia.
- Fast acclimation of methanogens at extremely high FAN levels (1633 mg NH_3-N L^{-1}).
- Hydrogenotrophic methanogens were dominant at FAN levels above 540 mg NH_3-N L^{-1}.
- CSTR acclimation failed at low TAN level (<4.6 g NH_4^+-N L^{-1}) due to washout effect.
- Fed-batch is a promising acclimation method to be coupled with bioaugmentation.

GRAPHICAL ABSTRACT



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Batch reactor
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ABSTRACT

Bioaugmentation with ammonia tolerant-methanogenic consortia was proposed as a solution to overcome ammonia inhibition during anaerobic digestion process recently. However, appropriate technology to generate ammonia tolerant methanogenic consortia is still lacking. In this study, three basic reactors (i.e. batch, fed-batch and continuous stirred-tank reactors (CSTR)) operated at mesophilic (37 °C) and thermophilic (55 °C) conditions were assessed, based on methane production efficiency, incubation time, TAN/FAN (total ammonium nitrogen/free ammonia nitrogen) levels and maximum methanogenic activity. Overall, fed-batch cultivation was clearly the most efficient method compared to batch and CSTR. Specifically, by saving incubation time up to 150%, fed-batch reactors were acclimatised to nearly 2-fold higher FAN levels with a 37%–153% methanogenic activity improvement, compared to batch method. Meanwhile, CSTR reactors were inhibited at lower ammonia levels. Finally, specific methanogenic activity test showed that hydrogenotrophic methanogens were more active than aceticlastic methanogens in all FAN levels above 540 mg NH_3-N L^{-1}.

1. Introduction

Anaerobic digestion (AD) is one of the most commonly used methods to treat a vast array of organic waste-slurries and wastewaters derived from different sources (e.g. agricultural waste, industrial waste, food waste, municipal sewage sludge etc.), which result in energy recovery (biogas; a mixture of CH_4 and CO_2) and in a nutrient-rich digestate used as biofertilizer (Bekkering et al., 2015). Additionally, AD reduces the greenhouse gas emissions and has lower energy requirements compared to other waste treatment methods (Westerholm et al., 2012). However, when ammonia-rich waste (e.g. animal manure, slaughterhouse wastewater etc.) are used as AD substrates, an instability

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Ammonia tolerant inocula provide a good base for anaerobic digestion of microalgae in third generation biogas process

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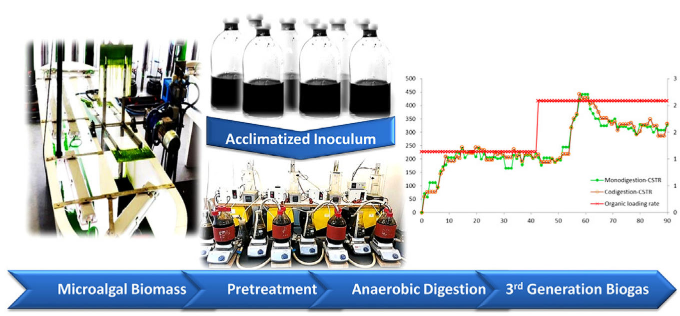
HIGHLIGHTS

- Efficient 3rd generation biogas production from protein-rich microalgae.
- Synergistic co-digestion occurred only when "algae VS"/"cattle manure VS" >1.
- High methane yield for algae achieved when ammonia tolerant inoculum was used.
- The highest yield was reached by coupling codigestion and ammonia tolerant inoculum.

GRAPHICAL ABSTRACT



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Ammonia inhibition
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ABSTRACT

This study investigated the ability of an ammonia-acclimatized inoculum to digest efficiently protein-rich microalgae for continuous 3rd generation biogas production. Moreover, we investigated whether increased C/N ratio could alleviate ammonia toxicity. The biochemical methane potential (BMP) of five different algae (*Chlorella vulgaris*)/manure (cattle) mixtures showed that the mixture of 80/20 (on VS basis) resulted in the highest BMP value (431 mL CH_4 g VS^{-1}), while the BMP of microalgae alone (100/0) was 415 mL CH_4 g VS^{-1}. Subsequently, anaerobic digestion of those two substrates was tested in continuous stirred tank reactors (CSTR). Despite of the high ammonium levels (3.7–4.2 g NH_4^+-N L^{-1}), CSTR reactors using ammonia tolerant inoculum resulted in relatively high methane yields (i.e. 77.5% and 84% of the maximum expected, respectively). These results demonstrated that ammonia tolerant inocula could be a promising approach to successfully digest protein-rich microalgae and achieve a 3rd generation biogas production.

1. Introduction

A great effort has been dedicated to the development of green technologies for producing renewable biofuels. Conversion of organic matter into biogas through anaerobic digestion (AD) has been intensely investigated since, unlike bioethanol or biodiesel, it holds the possibility to convert a broad variety of organic substrate to biogas (Naik et al., 2010). However, deeper understanding is required to address several challenges that can affect performance and stability of the conversion process when using new feedstocks. Microalgae, for example, seem to be a promising candi-

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Ammonia–LCFA synergetic co-inhibition effect in manure-based continuous biomethanation process

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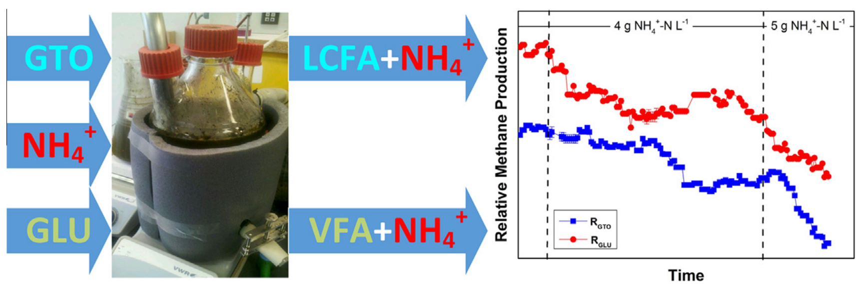
HIGHLIGHTS

- An "ammonia–LCFA synergetic co-inhibition" effect was identified.
- With lipids as co-substrates it is not possible to overcome ammonia toxicity on AD.
- Co-digestion with carbohydrate was more robust to ammonia toxicity than with lipid.
- Hydrogenotrophic archaea were more robust to ammonia–LCFA synergetic co-inhibition.

GRAPHICAL ABSTRACT



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Ammonia toxicity
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Lipids

ABSTRACT

In the current study it has been hypothesized that, when organic loading of an anaerobic reactor is increased, the additional cell biomass biosynthesis would capture more ammonia nitrogen and thereby reduce the ammonia toxicity. Therefore, the alleviation of the toxicity of high ammonia levels using lipids (glycerol trioleate-GTO) or carbohydrates (glucose-GLU) as co-substrates in manure-based thermophilic continuous stirred-tank reactors (R_{GTO} and R_{GLU}, respectively) was tested. At 5 g NH_4^+-N L^{-1}, relative methane production of R_{GTO} and R_{GLU}, was 10.5% and 41% compared to the expected uninhibited production, respectively. At the same time control reactor (R_{CTL}), only fed with manure, reached 32.7% compared to the uninhibited basis production. Therefore, it seems that using lipids to counteract the ammonia effect in CSTR reactors creates an "ammonia–LCFA (long chain fatty acids) synergetic co-inhibition" effect. Moreover, co-digestion with glucose in R_{GLU} was more robust to ammonia toxicity compared to R_{CTL}.

1. Introduction

Anaerobic digestion (AD) process, is a sustainable technology that is being used widely for organic waste treatment and concurrent bioenergy (i.e. biogas) production (Rajagopal et al., 2013). However, substrates with high nitrogen levels, such as slaughterhouse residues, pig and poultry manure, often result in inhibition of the AD process, due to release of ammonia during their degradation, which affects the stability and performance of the AD process in full-scale reactors (Moestedt et al., 2016). Ammonium ion (NH_4^+) and free ammonia (NH_3) are the two primary forms of inorganic ammonia nitrogen in aqueous solution (Chen et al., 2008). The mechanisms of ammonia inhibition in AD process have been thoroughly studied and NH_3 has been identified as the most toxic form of ammonia. In short, the passive diffusion of hydrophobic ammonia molecules into the microbes' cells may cause potassium deficiency, intracellular pH changes, increase maintenance energy requirements and suppress specific enzyme reactions (Sprott and Patel, 1986). The NH_3/NH_4^+ equilibrium is affected by the temperature, the pH and the total ammonia ($NH_4^+ + NH_3$) concentration in AD process (Rajagopal et al., 2013). In detail, as the pH and/or

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Research Article

RESEARCH ARTICLE

Ammonia effect on hydrogenotrophic methanogens and syntrophic acetate-oxidizing bacteria

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One sentence summary: SAOB were more sensitive to high ammonia compared to the hydrogenotrophic methanogens tested. Thus, hydrogenotrophic methanogens could be equally, if not more, tolerant to high ammonia levels compared to SAOB.

Editor: Alfons Stams

ABSTRACT

Ammonia-rich substrates can cause inhibition on anaerobic digestion process. Syntrophic acetate-oxidizing bacteria (SAOB) and hydrogenotrophic methanogens are important for the ammonia inhibitory mechanism on anaerobic digestion. The roles and interactions of SAOB and hydrogenotrophic methanogens to ammonia inhibition effect are still unclear. The aim of the current study was to determine the ammonia toxicity levels of various pure strains of SAOB and hydrogenotrophic methanogens. Moreover, ammonia toxicity on the syntrophic-cultivated strains of SAOB and hydrogenotrophic methanogens was tested. Thus, four hydrogenotrophic methanogens (i.e. *Methanoculleus bourgensis, Methanobacterium congolense, Methanoculleu thermophilus* and *Methanothermobacter thermautotrophicus*), two SAOB (i.e. *Tepidanaerobacter acetatoxydans* and *Thermacetogenium phaeum*) and their syntrophic cultivation were assessed under 0.26, 3, 5 and 7 g $NH_4{}^+$-N L^{-1}. The results showed that some hydrogenotrophic methanogens were equally, or in some cases, more tolerant to high ammonia levels compared to SAOB. Furthermore, a mesophilic hydrogenotrophic methanogen was more sensitive to ammonia toxicity compared to thermophilic methanogens tested in the study, which is contradicting to the general belief that thermophilic methanogens are more vulnerable to high ammonia loads compared to mesophilic. This unexpected finding underlines the fact that the complete knowledge of ammonia inhibition effect on hydrogenotrophic methanogens is still absent.

Keywords: ammonia inhibition; anaerobic digestion; biogas; SAOB; syntrophic growth

INTRODUCTION

Anaerobic digestion is a biological treatment for organic wastes by which pollution control and renewable energy can be obtained at the same time. Specifically, anaerobic digestion is a multistep process consisting of four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis, which are performed by different groups of microorganisms (Angelidaki *et al.* 2011). However, substrates that contain high total ammonia ($NH_4{}^+$ + NH_3) levels can inhibit the anaerobic digestion process and result in suboptimal biogas production (Fotidis *et al.* 2014). The unionized form of ammonia (free ammonia) is considered as the main toxic compound causing ammonia inhibition. Specifically, Sprott and Patel (1986) and Gallert, Bauer and Winter (1998) reported that passive diffusion of free ammonia into the microbes cells is causing proton imbalance, potassium deficiency, increase maintenance energy requirements and suppress specific enzyme reactions. Total ammonia concentration, temperature and pH affect free ammonia concentration in anaerobic digestion process (Chen, Cheng and Creamer 2008). Specifically, the shift from $NH_4{}^+$ to NH_3 is enhanced alongside the increase of pH and temperature and results in increased toxicity on the

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