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Title: Microbial adaptation to high ammonia levels during anaerobic digestion of manure-based feedstock; Biomethanation and 16S rRNA gene sequencing

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Abstract

BACKGROUND: Microbial consortia resistant to high ammonia concentrations may rescue biogas production from high ammonia containing wastes and manures during anaerobic digestion. Microbial communities were gradually enriched via sequential batch cultivations (stepwise exposure) at increasing ammonia concentrations up to the extremely high concentration of 9 g of total ammonia nitrogen (TAN) L^{-1}. This study examined the adaptation of anaerobic microbial consortia to high ammonia concentrations by the use of a complex substrate based on manure in order to generate tailor-made inocula for bioaugmentation purposes. RNA analysis was performed to determine the microbial community composition and activity of anaerobic bacteria and methanogens.

RESULTS: The enrichment series were successful in terms of methane production at ammonia concentrations as high as 5 g of TAN L^{-1} in comparison with the control concentration of 2 g of TAN L^{-1}. *Methanosarcina* was the core active dominant methanogen in all enrichment cultures and its relative activity was sharply increased at 3 g and 9 g of TAN L^{-1}. Cellulolytic bacteria activity mostly decreased with ammonia increase. Moreover, syntrophic butyrate and long-chain fatty acids degraders along with hydrogenotrophic methanogens were activated at increased ammonia levels.

CONCLUSIONS: The results of the study demonstrated an efficient method to enrich ammonia tolerant microbial consortia for bioaugmentation purposes in biogas reactors with concentrations up to 5 g of TAN L^{-1}. RNA analysis revealed high relative abundances of *Methanosarcina* and hydrogenotrophic methanogens at high concentrations of ammonia.

Key words: Anaerobic Digestion, Biochemical engineering, Biogas, Bioprocesses, Environmental Biotechnology, Microbiology.

Introduction

Methane production by anaerobic digestion (AD) can be strongly influenced by the presence of toxic compounds to microorganisms. Ammonia concentrations below 200 mg L^{-1} can be beneficial to AD, since nitrogen is essential for microbial metabolism,[1] yet, at high concentrations it can adversely influence AD. Total ammonia nitrogen (TAN) is found in the forms of free ammonia nitrogen (FAN-NH_3) and ammonium nitrogen (NH_4^+) which are in an equilibrium state in an aqueous solution (Eq1). The relative concentration of FAN-NH_3 and NH_4^+ depends on pH and temperature.[2] Of these two, free ammonia is considered as the main inhibitor.[1]

$$NH_3(aq.) + H_2O(l.) \leftrightarrow NH_4^+(aq.) + OH^-(aq.) \quad (1)$$

Past studies have been conducted for resolving the ammonia inhibition problem. Such methods include air-stripping,[3] waste co-digestion dilution,[4] chemical precipitation,[5] addition of absorbing material,[6] addition of soluble bio-based substances,[7] decreasing process temperature[8] and others. Despite the effectiveness of many of these methods against ammonia toxicity, they could be often economically unattractive or cumbersome.

Bioaugmentation with tolerant/resistant microbial enrichments has recently been demonstrated as a promising method for tackling ammonia inhibition in AD.[9] In this method, ammonia tolerant methanogenic cultures are injected into anaerobic reactors leading to an alteration of the microbial composition towards increased tolerance to high ammonia loads.[10] Generation of ammonia tolerant inocula has been previously reported.[11,12] Bioaugmentation to tackle ammonia toxicity can be either performed by the use of specific microbial strains[13] or adapted mixed microbial consortia.[14] Mixed over pure cultures are preferred because they are more robust and they can be easily adapted to specific environmental conditions possibly due to functional diversity and synergistic interactions.[15,16]

It has been well established that methanogens are more sensitive to high ammonia levels than other groups of microorganisms that participate in the first steps of AD process.[17] Hydrogenotrophic methanogens were reported to be more resilient under ammonia toxicity than aceticlastic methanogens,[18] with literature, however, being inconsistent.[19] In most of the previous

studies, simple methanogenic substrates like acetate were used as a carbon source for enriching ammonia-tolerant methanogens for bioaugmentation purposes. In this way, ammonia tolerant acetate utilizing microbial consortia and aceticlastic methanogens were specifically selected.[9,20] However, functional groups beyond methanogens are also affected by ammonia. Niu *et al.* reported inhibition of protein and carbohydrate degradation at TAN concentrations of 3 and 4 g L^{-1}, respectively.[21] Assuming that ammonia toxicity is not limited to methanogens, adapted inocula should be functionally expansive beyond methanogenesis. Complex substrates may provide the foreground for a microbial community-wide adaptation to ammonia.

Some earlier studies have examined the effect of increased ammonia concentration on the microbial dynamics with the use of complex substrates via DNA-based analysis.[10,22] Although the microbial community composition analysis by DNA screening provides good insights on the composition of the tested microbial communities, RNA analysis can provide accurate snapshots of the active microbiome in dynamic systems like AD under ammonia stress.

The main aim of this study was the adaptation of microbial consortia to high levels of ammonia during anaerobic digestion of a manure-based feedstock in order to generate tailor-made microbial inocula for bioaugmentation purposes. The performance of the enriched cultures exposed to stepwise increasing ammonia levels was assessed on basis of methane production at various ammonia concentrations in batch anaerobic reactors. Determination of the anaerobic

microbiome changes during its adaptation at increased ammonia concentration is important for the optimization of the enrichment process of ammonia tolerant microbial consortia for bioaugmentation purposes. Unlike previous studies, a complex substrate –based on manure was used to adapt microbial consortia at high ammonia concentrations. Furthermore, in this study, RNA analysis was performed in each adaptation step, providing a more insightful and detailed understanding of the changes occurring during enrichment in the entire active anaerobic microbial communities (bacteria and archaea), in contrast with most of the previous studies that examined only the present communities, to further optimize the bioaugmentation process.

Experimental

Feedstock and inoculum

Samples from different waste streams of a full-scale mesophilic biogas plant-Biogas Lagadas (Central Macedonia, Greece) were obtained and stored at -21°C. All the samples were thawed at 4°C for 3 days before use. The final feedstock was created by mixing the waste samples according to data obtained from Biogas Lagadas and it consisted of 71.8% cow manure, 12.7% poultry manure, 7.8% whey, 2.8% pig slurry, 2.1% fruit pulp, 2.0% maize silage and 0.8% spent wine grapes in percentage of weight. The inoculum was obtained from the same biogas plant and it was pre-incubated under anaerobic conditions at a temperature of 37±1°C for 7 days in order to reduce its residual CH_4 production. The characteristics of inoculum and final substrate used in the AD batch experiments are depicted in Table 1.

Experimental design

Microbial enrichment was performed by successive batch cultivations, where new batch reactors containing higher ammonia concentrations were inoculated from the previous reactors of lower ammonia load. Each of these enrichments cultures were named with a number and the letters NS, where the number represented the desired nominal concentration of TAN in g L^{-1} i.e. 2NS was the batch culture with the nominal concentration of 2 g L^{-1} of TAN. The inoculum for the next step was obtained when the CH_4 production of the previous lower ammonia culture was at the exponential phase. The batch cultures were carried out in 1131.5 mL glass reactors and with a working volume of 280 mL. Each reactor was filled with inoculum (140 mL), feedstock (70 mL) and a water solution of NH_4Cl (70 mL). The content of NH_4Cl in the solution was adjusted at each step to achieve the desired concentration of TAN L^{-1}. Batch cultures of the stepwise exposure procedure are presented in detail in Table 2.

The effect of the growth stage (exponential or stationary) for retrieving inoculum for the next batch cultivation on the adaptation of microbial consortia in terms of CH_4 production to increased ammonia levels was also examined. For this reason, an additional culture with 4 g of TAN L^{-1} was included, using inoculum from the 3 g of TAN L^{-1} batch culture obtained at the stationary CH_4 production phase. This new batch culture was named 4NSS where the number represented also the desired nominal concentration of TAN in g L^{-1} and the letters NSS were used for distinguishing this culture from the rest cultures where inoculum was obtained at the exponential CH_4 production phase. The total time needed to adapt microbial consortia at high

ammonia levels during the stepwise process is important for the applicability and economic attractiveness of this process[9] and it is affected by the inoculum growth phase derived from the previous ammonia level culture.

All batch reactors were flushed with a gas mixture of N_2/CO_2 (80/20, v/v) to create anaerobic conditions, incubated at 37 ± 1°C and they were vigorously mixed, manually, once per day. All batch cultures were performed in triplicate. Three additional blank reactors were used in each step of ammonia level to monitor the inoculum CH_4 production, which was subtracted from the production of batch cultures.

Analytical methods

Total solids (TS), volatile solids (VS), Total Kjeldahl Nitrogen (TKN) and TAN were determined according to Standard Methods.[23] The pH value of the solutions was measured with a bench digital pH meter (JENWAY 3520, Essex, UK).

A gas tight syringe equipped with a pressure lock and an attached needle was used to obtain samples from the headspace of the reactors. Biogas production was monitored daily via injection of a gas sample into a gas chromatograph (GC-2010plusAT, SHIMADZU, Kyoto, Japan) equipped with a thermal conductivity detector (GC-TCD) and two columns: (a) GC-Column, FS, ValcoPLOT HayeSep D (15 m x 0.53 mm i.d. and 20 μm film thickness) and (b) VP-Molesieve

Column, FS, ValcoBond, 5A Fused Silica (15 m x 0.53 mm i.d. and 20 μm film thickness). The biogas injected volume was 150 μL and the temperature of the columns was constant at 31.5°C. Volatile fatty acids (VFAs) were analyzed in a gas chromatograph (GC-2010plusAT, SHIMADZU, Kyoto, Japan) equipped with a flame ionization detector (GC-FID) and a ZB-FFAP column, Zebron-Phenomenex (30 m x 0.53 mm i.d. and 1 μm film thickness). Column oven temperature program was set for the first 3.5 min at 60°C, with temperature increase at 25°C/min to 130°C and then 10°C/min to 235°C and final hold of 5 min. The injection sample volume for the measurement of VFAs was 1 μL. Helium was used as carrier gas in the gas chromatographs.

Nucleic acids extraction, 16S rRNA gene amplicon sequencing and bioinformatics

In the present study, nucleic acids (DNA and RNA) were extracted from bioreactor samples (triplicate) when CH_4 production was at the exponential phase of successive bioreactor inoculations for microbial community analysis. Samples for analysis of the original inoculum were retrieved just after inoculation of the first batch culture. DNA and RNA were co-extracted with the NucleoBond® RNA Soil kit and its DNA co-ellution set (Macherey Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer's instructions. RNA reverse transcription to cDNA (RNA) was carried out with the PrimeScriptTM RT Reagent Kit (Takara, Kusatsu, Shiga Prefecture, Japan). The bacterial and archaeal 16S rRNA gene analysis was performed via multiplex amplicon sequencing as detailed in our previous study,[24] using DNA extracts or cDNA

as templates in the two-step polymerase chain reaction (PCR) based process. The primers used were those suggested by Earth Microbiome Project consortium,[25] namely the 515f-806r [26,27] set (515f 5'-GTGYCAGCMGCCGCGGTAA-3', 806r 5'-GTGYCAGCMGCCGCGGTAA-3'). The multiplexed PCR products were sequenced using the TruSeq Nano DNA kit (Illumina, San Diego, CA, USA) for the library preparation and an Illumina HiSeq 2500 sequencer at the Brigham Young University sequencing facilities (Provo, UT, USA), generating 250bp paired-end reads. The 0.03 distance-defined operational taxonomic units (OTUs) matrices were obtained with the OptiClust algorithm[28] after removal of sequencing/PCR artifacts as described in Vasileiadis *et al.*[24] The amplicon sequence reads used for microbiome analysis performance were submitted at the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) and are publicly available under the Bioproject number PRJNA592718.

Statistical analysis

Statistical analysis of the data of the characteristics of inoculum and substrate, CH_4 production, and the maximum specific growth rate was performed with the IBM SPSS Statistics, version 25. Mean and standard deviation values were calculated, as also comparisons of the means were performed by one-way analysis of variance, and the pair-wise differences were assessed with the Tukey's post hoc analysis (statistical significant difference if $p < 0.05$). Concerning the microbial community analysis, the achieved coverage by the employed sequencing effort and α-diversity indices was calculated with the Entropart v1.5-3[29] and the Vegan v2.5-4[30] packages of the R

v3.5.1[31] software with Vegan being used also for the multivariate approaches. The α-diversity indices were calculated in a defined community of OTUs with a relative abundance of at least 0.1%. Analysis of variance (ANOVA) and a Tukey's post-hoc analysis or the non-parametric equivalents of the Kruskal-Wallis test followed by the Wilcoxon rank sum test were implemented for assessing statistically significant differences between the cultures for the various tested habitats and time-points using the Agricolae v3.5.2 package[32] of the R software. The data were further transformed using the Hellinger transformation[33] and were used for performing principal components analysis and its canonical version, namely, redundancy analysis, with the latter being selected according to the 1st axis length value of detrended correspondence analysis being lower than 3 standard deviations as previously suggested.[34] The permutational analysis of variance (PERMANOVA) tests[35] accompanying redundancy analysis (RDA)[36] were performed using 999 permutations. The non-parametric Kruskal-Wallis test followed by the Wilcoxon rank sum test (if the occasions where Kruskal Wallis test was significant) was used for identifying differentially abundant/expressed OTUs among the batch cultures as previously suggested.[37]

Calculation of maximum specific growth rate (μmax)

The maximum specific growth rate of the methanogens (μmax) was calculated from the slope of the linear part of the graph of methane production natural logarithm versus time.[20]

Calculation of free ammonia nitrogen (FAN)

The FAN concentrations were calculated by the following equation:

$$FAN = \frac{TAN}{1 + \frac{10^{-pH}}{K_a}}$$

Where TAN is total ammonia nitrogen of the solution, pH is the pH value of the solution and K_a is the dissociation constant which at mesophilic conditions (37 °C) is equal to 1.29 x 10^{-9}.[3]

Results and discussion

Methane production of stepwise exposure and adaptation of microbial consortia

The accumulative CH_4 productions of each batch culture is given in Fig. 1A, while the CH_4 production in mL of CH_4 g^{-1} VS added for 30, 60 and 120 days is presented in Fig. 1B. Batch culture 2NS was used as a reference - control for uninhibited CH_4 production (Fig. 1B), since NH_4Cl was not added and the TAN level in those batches was approximately 2 g L^{-1}, a concentration considered not toxic. The CH_4 production of the reference – control batch culture of 2NS for the first 30 days period was 1591.50 mL of CH_4 (accumulative production) and 271.22 mL of CH4 g^{-1} VS added. The accumulative CH_4 production curve of the cultures up to 5 g of TAN L^{-1} followed the same trend with the reference culture and no statistical differences of the CH_4 production (mL CH_4 g^{-1} VS added) of these cultures were observed for a 30 days period. However, at cultures of 6 g and 9 g of TAN L^{-1} (6NS and 9NS) the CH_4 production was significantly lower. These results strongly indicate that microbial consortia were only adapted successfully to up to 5 g of TAN L^{-1}. In order to elucidate whether prolonged cultivation could

promote adaptation of the microbes exposed to high ammonia concentrations, the AD process was followed for additional 120 days in total. Nevertheless, the CH_4 production of the 6NS and 9NS cultures never reached the methane production of the control probably due to high ammonia concentrations (Fig. 1B).

The maximum specific growth rate of each culture of the stepwise exposure to high concentrations of ammonia is presented in Fig. 2. The μmax values ranged between 0.022 and 0.028 h^{-1}. The highest value was observed at 5 g of TAN L^{-1} culture and the lowest at 9 g of TAN L^{-1} culture. The μmax values at 4, 5 and 6 g of TAN L^{-1} cultures were quite similar (no statistical difference), and a significant drop was observed at 9 g of TAN L^{-1}. Increase of μmax with the increase of ammonia levels up to 5 g of TAN L^{-1} during the enrichment process of a mesophilic inoculum, was also reported in a previous study.[16] Similar fluctuation and range of μmax values were observed when mesophilic inoculum was stepwise exposed to increasing ammonia concentrations from 1 to 7 g TAN L^{-1}.[20] Doubling time of the methanogenic consortia in all cultures ranged between 1.03 and 1.31 days. These results indicate that all the cultures, even the culture of 9 g of TAN L^{-1} (9NS), can be used as inoculum in a bioaugmentation procedure of continuously fed reactors, typically operated at hydraulic retentions times (HRTs) of over 20 days, without the risk of being washed out. Therefore, adaptation of microbial consortia to high levels of ammonia by the stepwise ammonia increase was a successful approach to generate robust to ammonia bioaugmentation cultures.

Impact of growth phase for inoculation on stepwise exposure adaptation

The accumulative CH_4 production of cultures 4NS (inoculum derived at exponential phase) and 4NSS (inoculum derived at stationary phase) for a period of 30 days are presented in Fig. 3. The total CH_4 production was almost the same after 30 days between the cultures and a similar microbial diversity was observed in the microbial analysis of α and β-diversity (See section 3.3.1, Fig 4). Methane production of 4NS and 4NSS was 271.73 and 261.46 mL of CH_4 g^{-1} VS added. Except these similarities, the upward trend of the CH_4 production in 4NS culture was sharper than 4NSS for the first days of the AD (Fig. 3). This result indicates that the microbial consortia obtained at exponential phase were adapted in less time in comparison with the microbial consortia derived at stationary phase. Moreover, the time needed for methane production to reach the exponential phase is shorter than the time needed to reach stationary phase. Based on these results, the preferred choice to retrieve the inoculum for the next step of the stepwise increase of ammonia levels for adapting microbial consortia to these conditions is the exponential phase.

Impact of ammonia on microbial communities

DNA and RNA analysis were performed for all batch cultures of the stepwise exposure process. In this section only the RNA analysis is discussed as it provides more accurate snapshot of the active microbiome, while DNA analysis results are presented as Supplementary Material.

Microbial α and β Diversity

As shown in Fig. 4A, with the increase of ammonia up to 6 g of TAN L^{-1}, the microbial diversity (α-diversity) of the cultures increased, possibly resulting to a higher functional diversity and flexibility of the microbial consortia. An increase of the microbial diversity was also reported at 4 - 6 g of TAN L^{-1}, in a continuously stirred tank reactor (CSTR) fed with chicken manure, compared with the diversity at 2 - 3.5 g of TAN L^{-1}.[38] At 9 g of TAN L^{-1}, the microbial diversity dropped significantly, possibly showing a selection for more tolerant OTUs to ammonia toxicity.

The initial inoculum and cultures associated structural differences coinciding with a high proportion of the observed variance (90.3% and model being significant; P 0.001), and RDA1 and RDA2 encompassing 56.2% and 19.3% of the canonical variance, respectively (Fig. 4B). The β-diversity analysis of the community structures suggested three-four major groupings for the RNA templates. Overall, a stepwise-dependent evolution of the communities was observed considering their composition. The cultures of 3 to 6 g TAN L^{-1} seemed to be structurally quite similar, while a pronounced difference was observed between them and the 9 g of TAN L^{-1} culture, indicating that the final microbiome compositions were differentially affected by ammonia contents.

Considering the 4NS (inoculum derived at exponential phase) and 4NSS (inoculum derived at stationary phase) cultures shared a similarity in terms of β-diversity and no statistical differences

were observed at all α-diversity indices. Therefore, the growth phases for inoculation were indifferent to microbial diversity whether the inoculum was retrieved during the exponential or stationary phase.

Overall OTU classification and differential abundance among batch cultures

The total population sequences obtained from the RNA analysis were assigned to 96.64% to bacterial OTUs and 3.36% to archaeal OTUs. In general, from the bacterial sequences of RNA analysis a majority were classified into two phyla: *Firmicutes* (43.0%) and *Bacteroidetes* (30.3%). These two phyla were also frequently detected in microbial consortia of AD experiments reported by other researchers.[39] The dominant phylum of the archaeal community was *Euryarchaeota* with 98.65% of the active OTUs from the archaeal sequences classified to this phylum. Less than 1% of the rest archaeal sequences where classified within the phyla of *Crenarchaeota* and *Thaumarchaeota*.

Biofiber degraders affected by high ammonia concentrations

Active populations of cellulolytic bacteria with increased relative abundance were observed in all stepwise exposure enrichments. The OTU10 was associated to species level to *Fibrobacter succinogenes* (BLASTn NCBI database identity: 90%), which is one of the major cellulolytic bacteria in the rumina of cattle and sheep that produce primarily succinic and acetic acid.[40] The representative sequence of the OTU42 with an identity of 93% to *Hungateiclostridium*

clariflavum (BLASTn NCBI database) can also utilize cellulose.[41] Ammonia nitrogen concentration over 3 g of TAN L^{-1} had a strong influence on the active communities of the *F. succinogenes* and *H. clariflavum* as their relative abundance sharply declined in comparison with the enrichment of 2 g of TAN L^{-1}. This strongly suggests that the high ammonia concentration reduced the cellulosic hydrolysis and may negatively affect the CH_4 production. However, OTU47 associated with the genus *Cellulosilyticum* and at species level with *Cellulosilyticum ruminicola* (BLASTn NCBI database identity: 93%) increased significantly its relative activity up to the concentration of 4 g of TAN L^{-1}, and maintained this high level of activity in the subsequent cultures of higher ammonia concentrations. *C. ruminicola* can utilize cellulose and xylan as carbon energy sources and it produces formic acid, acetic acid, CO_2 and trace amounts of ethanol, lactic acid and succinic acid.[42] Moreover, genus *Ruminofilibacter* (OTU2), from the phylum *Bacteroidetes* was found as one of the most dominant genera in all cultures. The representative sequence OTU2 was searched with BLASTn against NCBI database and showed a 93% identity to *Alkaliflexus imshenetskii*, which was reported as anaerobic saccharolytic bacteria able to degrade xylan.[43] The aforementioned predominant genera probably contributed significantly to the stabilization of the anaerobic digestion and resulted to an increased methane production.

Bacteria and archaea syntrophic interactions for methane production

RNA analysis revealed active dominant prokaryotic OTUs that could syntrophically interact with hydrogenotrophic methanogens. Five from the one hundred most dominant OTUs resulted from the RNA analysis were members of the *Syntrophomonas* genus. The representative sequence annotations of these OTUs were validated and closest species were identified using BLASTn (16S rRNA NCBI collection). OTU14 was associated to *S. zehnderi* (95% identity), OTU29, OTU71 and OTU83 to *S. bryantii* (96-97%), OTU81 to *S. wolfei* (97%) and OTU94 to *S. sapovorans* (97%). All of these species have been previously described to degrade butyrate, as well as some long-chain fatty acids (LCFAs) (up to C_{18}) in syntrophic association with hydrogenotrophic methanogens.[44] Relative activity of all the above OTUs related to *Syntrophomonas* genus increased significantly in all cultures in comparison with the initial inoculum. Moreover, similar fluctuations of relative activity in all cultures were observed between OTU4 and *S. zehnderi* and *S. bryantii*. OTU4 was associated with the species *Clostridium chauvoei* (BLASTn, identity: 98%), which is able to produce acetate and butyrate from pyruvate[45] and possibly a relation between these prokaryotic bacteria existed.

The most predominant archaeal OTU found active in all batch cultures was taxonomically assigned at species level as *Methanosarcina flavescens* (OTU13, BLASTn, identity: 99%). *M. flavescens* was a recently reported methanogen, isolated from a full-scale biogas plant and it can grow and produce CH_4 by the aceticlastic and hydrogenotrophic pathway as well as methylotrophically with methanol, mono-, di-, and trimethylamine.[46] Relative abundance of 16S

rRNA gene transcripts of *M. flavescens* was escalated at batch cultures of 3 g and 9 g of TAN L^{-1} (Fig. 5), which indicated an increased activity at lower concentrations of ammonia but also a tolerance to a high level of ammonia toxicity. Similar results were reported by Dai *et al*. where an acute inhibition on *Methanosarcina sp*. was observed at concentrations above 3 g of TAN L^{-1} and fully recovered at concentrations of 5 - 6 g of TAN L^{-1}.[47] *Methanosarcina sp.* interacts with syntrophic acetate-oxidizing bacteria (SAOB) and could act as an aceticlastic and as a hydrogenotrophic methanogen in low and high ammonium concentrations and organic loading rates, respectively.[48] Furthermore, BLASTn search (16S rRNA NCBI collection) showed that OTU57 was related to (99% identities) *Methanoculleus bourgensis*, which was the third most active methanogen. The recent complete genome sequence of *M. bourgensis* suggested it to be a suitable hydrogenotrophic methanogenic partner for mesophilic SAOB *Clostridium ultunense*, *"Tepidanaerobacter acetatoxydans"* and *Syntrophaceticus schinkii*.[49] None of the above SAOB were found by the RNA analysis at increased TAN concentrations in high relative abundance. However, an increased relative activity of *M. bourgensis* was observed at the cultures of 3 g and 9 g of TAN L^{-1}, which was in relevance with the increased activity of syntrophic butyrate and LCFAs degraders *S. zehnderi* (OTU14) and *S. sapovorans* (OTU94), respectively. Moreover, increased relative activity was observed from the *M. bourgensis* and *S. bryantii* (syntrophic butyrate and LCFAs degrader - OTU29) at the cultures of 6 g and 9 g of TAN L^{-1}. It seems that the activity of *M. bourgensis* is probably affected by the activity of the syntrophic associated bacteria. Active bacterial populations able to produce and syntrophically degrade butyrate and

LCFAs were dominant in all cultures and in relation to the increased activity of *M. bourgensis* (the most dominant hydrogenotrophic methanogen) and *M. flavescens*, it was concluded that CH_4 production through the hydrogenotrophic pathway occurred mainly at high ammonia levels in the present study.

Hydrogenotrophic methanogens from the genera *Methanobrevibacter* (OTU115) and *Methanocorpusculum* (OTU129) were found with active sub-populations in all cultures. The genus *Methanobrevibacter* had non-significant fluctuations of its relative activity with the increase of ammonia concentrations, however a slightly increased relative activity was observed at concentrations up to 3 g and 9 g of TAN L^{-1}. The relative activity of *Methanocorpusculum,* which was third hydrogentotrophic methanogen in relative abundance, decreased significantly at 3 g of TAN L^{-1}.

Hydrogenispora ethanolica was associated via BLASTn 16S rRNA NCBI collection (identity: 89%) with OTU3 which was highly active in all batch cultures and it was reported that it had a potential syntrophic interaction with the hydrogenotrophic methanogens.[50] Moreover, *Acetomicrobium hydrogeniformans* was identified (97%) from the consensus sequence of the OTU11 and it could produce H_2 from glucose and grow syntrophically with hydrogenotrophic methanogens.[51] Archaea able to produce CH_4 through the hydrogenotrophic pathway were found

active in all batch cultures (Fig. 5) and probably had a syntrophic interaction with the aforementioned prokaryotic bacteria.

Methylotrophic methanogens affected by the increased ammonia concentrations

The second most active methanogen (OTU41) was assigned to *Methanomassiliicoccus luminyensis* (BLASTn NCBI collection 96% identity). *M. luminyensis* strains can use H_2 as an electron donor and reduce methanol to CH_4.[52] Relative abundance of 16S rRNA gene transcripts of *M. luminyensis* was reduced in all cultures in comparison with that observed at the initial inoculum but remained present in all cultures (Fig. 5). *M. luminyensis* was found as a dominant methanogen of the archaeal community of biogas reactors in previous studies of ammonia toxicity.[16,53] Except for *M. luminyensis*, four additional OTUs (OTU70, OTU72, OTU87 and OTU104) were taxonomically affiliated to methylotrophic archaea. In general, the relative activity of these OTUs was very low at the initial inoculum and increased significantly with the increase of ammonia concentrations. However, the relative activity of the OTU87 assigned to the genus *Methanomicrococcus* decreased significantly at the extreme concentration of 9 g of TAN L^{-1} and reached the low level of activity observed at the initial inoculum. Methylotrophic archaea detected in this study were in high relative abundance in all batch cultures, and probably had a significant role in CH_4 production.

Conclusions

The results obtained of this study showed that microbial adaptation with stepwise exposure was successful up to 5 g of TAN L^{-1}. The preferred choice to retrieve the inoculum for the next step is the exponential rather than the stationary phase. RNA analysis showed that *M. flavescens* was the most active methanogen in all cultures and increased its relative abundance at the high level of 9 g of TAN L^{-1}. Activity of syntrophic butyrate and LCFAs degraders increased significantly alongside with hydrogenotrophic methanogens at increased ammonia levels. Methylotrophs high relative abundance among the methanogens in all cultures strongly indicates that they had a significant role in CH_4 production.

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Table 1: Characteristics of substrate and inoculum.

Parameter	**Inoculum** (Value±SD*)	**Substrate** (Value±SD*)
Total Solids, TS (g L^{-1})	42.62±1.29	99.78±0.22
Volatile Solids, VS (g L^{-1})	31.28±1.01	83.83±1.00
Total Kjeldahl Nitrogen, TKN (mg N L^{-1})	4446±32.16	5304±55.68
Total Ammonia Nitrogen, TAN (mg N L^{-1})	3277±23.70	3034±31.85
Free Ammonia, FAN (mg NH_3-N L^{-1})	251.92±1.82	248.57±2.61
Volatile Fatty Acids, VFAs (g L^{-1})	1.23±0.01	34.11±3.40
pH	7.81±0.07	7.84±0.04

* Standard Deviation

Table 2: Batch cultures of the stepwise exposure and total ammonia nitrogen (TAN) concentrations.

Culture	Initial Inoculum	Inoculum origin at stepwise enrichment	Substrate	NH_4Cl Added	Nominal TAN (mg L^{-1})	Measured TAN (mg L^{-1}) (Value±SD*)
2NS	+	-	+	-	2000	2383.95 ± 191.52
3NS	-	2NS (exp†)	+	+	3000	3198.42 ± 103.55
4NS	-	3NS (exp)	+	+	4000	4143.87 ± 102.63
5NS	-	4NS (exp)	+	+	5000	5008.16 ± 41.6
6NS	-	5NS (exp)	+	+	6000	6029.15 ± 36.25
9NS	-	6NS (exp)	+	+	9000	9565.01 ± 193.6
4NSS	-	3NS (stat**)	+	+	4000	4134.62 ± 101.97

* Standard Deviation, † Exponential phase, ** Stationary phase

Figure captions

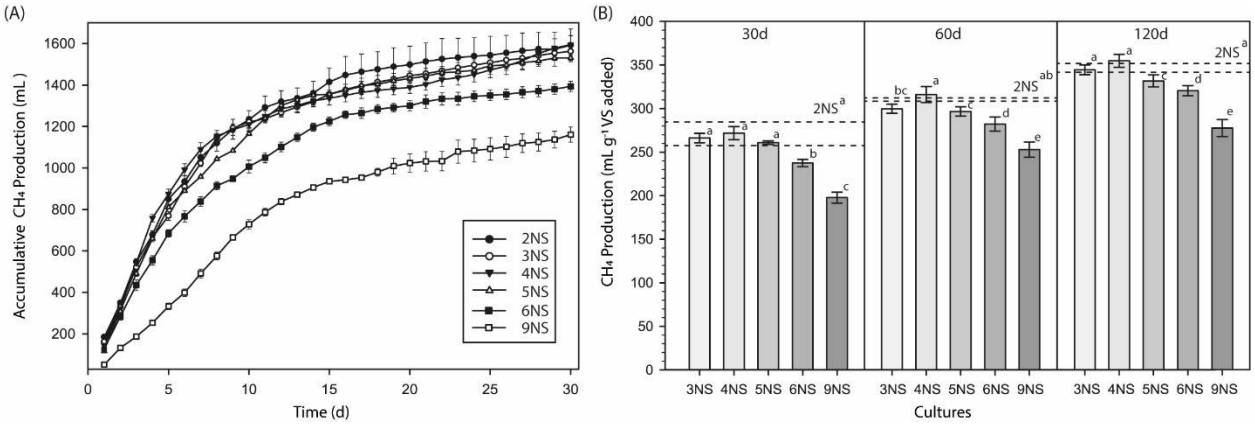
Fig. 1. Stepwise exposure to increasing ammonia loads. A) Accumulative CH_4 (mL) production in 30 days and B) CH_4 (mL g^{-1} VS added) production in 30 days, 60 days and 120 days. Different letters above the bars signify distinct statistical groups ($p < 0.05$) between the different cultures.

Fig. 2. Maximum specific growth rate (μmax) of cultures with different ammonia concentration levels. Different letters above the bars signify distinct statistical groups ($p < 0.05$) between the different cultures.

Fig. 3. Accumulative methane production (mL) of cultures 4NS and 4NSS. Data are expressed as the mean ± standard deviation.

Fig. 4. (A): α-diversity indices for the cDNA (RNA) templates associated with the activity of the identified OTUs. Different letters beside the bars signify distinct statistical groups ($p < 0.05$) between the different cultures. (B) Redundancy analysis scatter plot of the samples according to their microbial community compositions and using the culture (inoculum) factor as constraining variable for the cDNA samples. Ellipses indicate the culture-wise sample groupings. The model coinciding variance and the associated permutation test (999 permutations) P-value are provided on top of the plot. Grey arrow directions indicate the gradients of the 13 most dominant OTUs throughout the samples while the arrow length is linked to the abundance of the corresponding OTU in the samples towards the arrow's direction.

Fig. 5. Heatmap of the cDNA (RNA)-based relative abundances scaled from 0 to 1 (see grey to orange key) for each OTU throughout all samples of the 50 most dominant prokaryotic and 9 archaeal OTUs. The mean relative abundance of each OTU throughout all samples is indicated by the white-to-green column at the left according to the secondary key. Hierarchical clustering with the average linkage algorithm was performed for depicting sample and OTU groupings.



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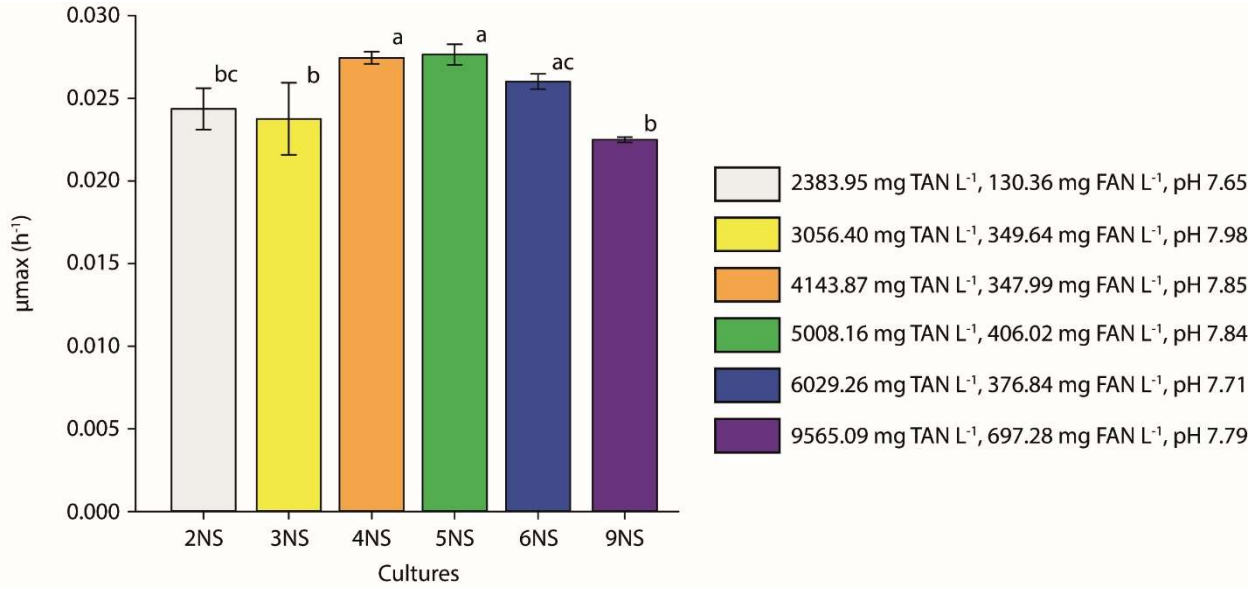

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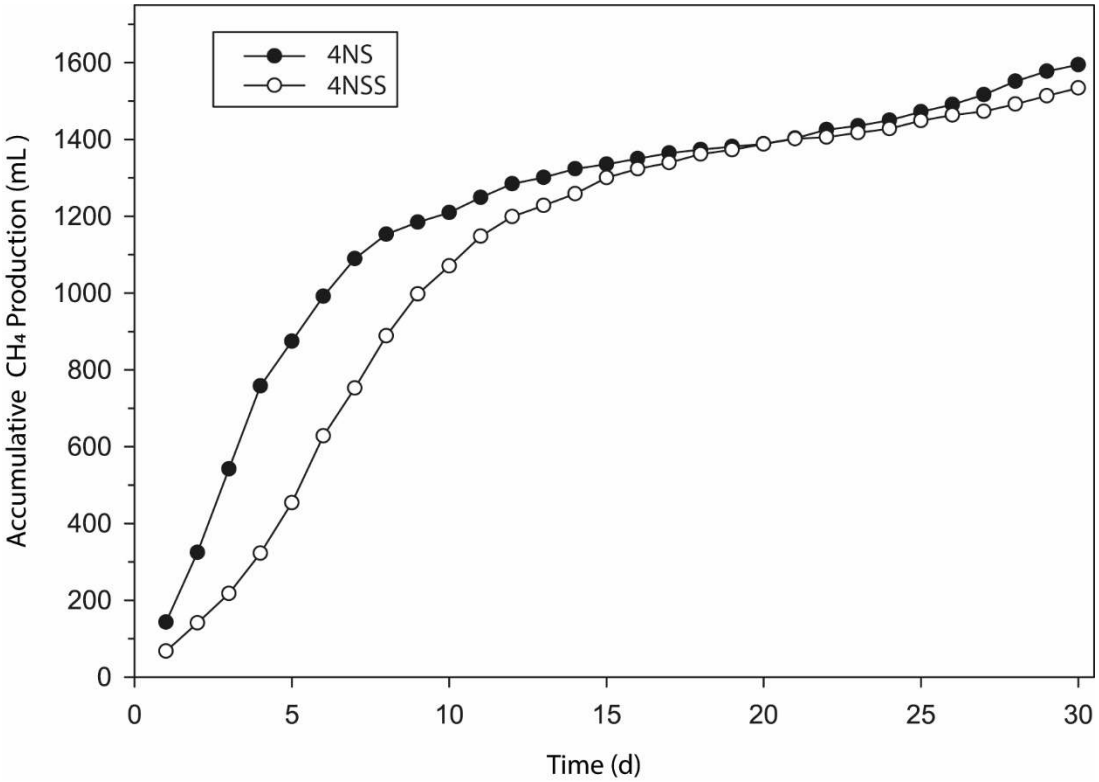

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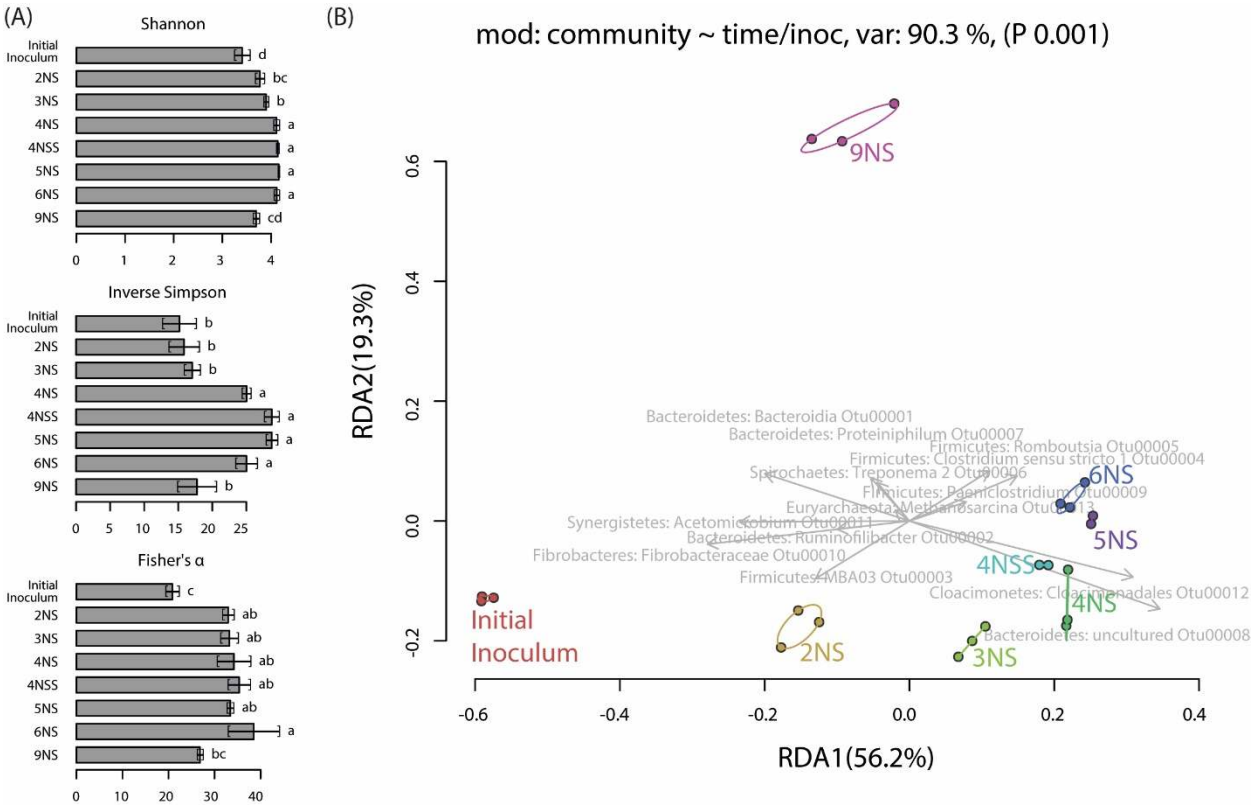

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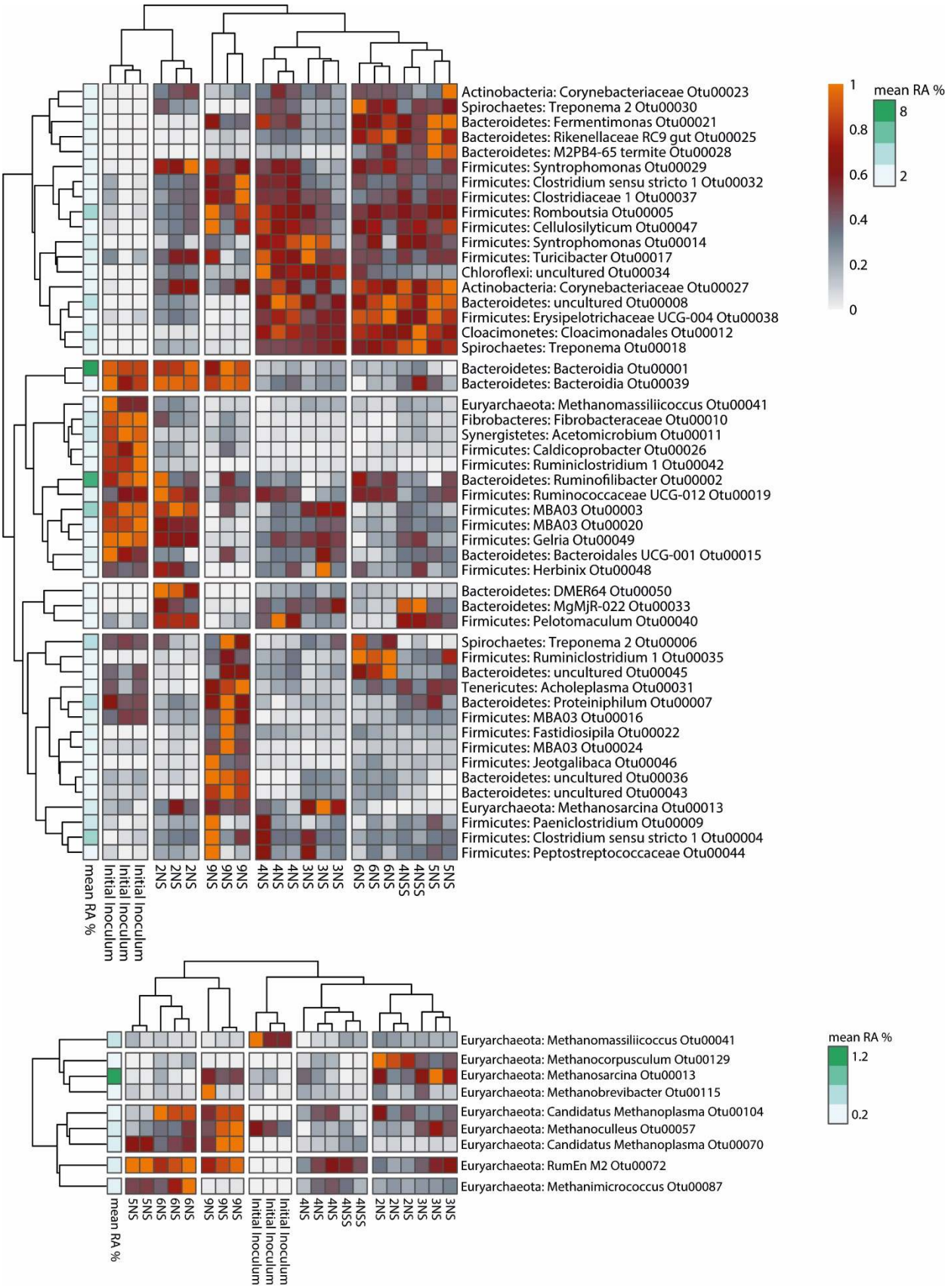

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