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

Hailin Tian ^a, Ioannis A. Fotidis ^{a,*}, Enrico Mancini ^a, Laura Treu ^{a,b}, Ahmed Mahdy ^c,
Mercedes Ballesteros ^{d,e}, Cristina González-Fernández ^d, Irini Angelidaki ^a

^a Department of Environmental Engineering, Technical University of Denmark, Bygningstorvet
Bygning 115, DK-2800 Kgs. Lyngby, DK

^b Department of Agronomy, Food, Natural Resources, Animal and Environment (DAFNAE),
Viale dell'Università, 16, 35020 Legnaro (Padova), Italy

^c Department of Agricultural Microbiology, Faculty of Agriculture, Zagazig University, 44511
Zagazig, EG

^d Biotechnological Processes for Energy Production Unit – IMDEA Energy, 28935 Móstoles,
Madrid, ES

^e Biofuels Unit – Research Center for Energy, Environment and Technology (CIEMAT), 28040
Madrid, ES

*Corresponding Author: Ioannis A. Fotidis, Department of Environmental Engineering,
Technical University of Denmark, Bygningstorvet Bygning 115, DK-2800 Kgs. Lyngby,
Denmark, Phone: (+45) 45251418; Fax: (+45) 45933850; e-mail: ioanf@env.dtu.dk

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 $10 \text{ g NH}_4^+\text{-N L}^{-1}$, was performed in mesophilic ($37\pm 1^\circ\text{C}$) 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 ($>7 \text{ g NH}_4^+\text{-N L}^{-1}$). 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.

Keywords

Methane; ammonia inhibition; microbial community; syntrophic acetate oxidizer; *Methanosarcina*.

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) acetoclastic pathway and b) hydrogenotrophic pathway (syntrophic acetate oxidation (SAO) coupled with hydrogenotrophic methanogenesis). Acetoclastic pathway is mediated by *Methanosarcinaceae* spp. and *Methanosaetaceae* spp., while *Methanomicrobiales* spp., *Methanobacteriales* spp., *Methanococcales* spp., *Methanopyrales* spp. and *Methanocellales* spp. mediate the hydrogenotrophic partway (Lyu & Lu, 2015). Furthermore, there is also evidence showing that some members of *Methanosarcinaceae* spp. can perform both acetoclastic and hydrogenotrophic pathways (Liu & Whitman, 2008).

Many compounds (e.g. ammonia, sulphide, light metal ions, heavy metals, organics etc.) can affect the AD microbial community, cause reactor instability with low methane yield. Ammonia is the major toxicant of the commercial AD reactors and usually derives from the degradation of urea and protein-rich substrates, such as slaughterhouse wastewater and food waste etc. Latterly, microalgae, a 3rd generation biomass, has been considered as biomass for biogas production, because it does not compete with food supply and has a high methane potential. However, the use of protein-rich microalgae as AD substrate has been proven to be very difficult due to its high nitrogen content (Maity et al., 2014). Total ammonia (TAN) is presented as ammonium ion (NH_4^+) and free ammonia (NH_3 , FAN) depending on the pH and temperature of the aqueous phase. FAN is believed to be the most toxic form of TAN due to its high permeability into cell membrane (Massé et al., 2014). Different inhibition thresholds were reviewed in literature (Yenigün & Demirel, 2013). However, it is generally accepted nowadays that

TAN and FAN loads above $3 \text{ g NH}_4^+-\text{N L}^{-1}$ and $0.15 \text{ g NH}_3\text{-N L}^{-1}$ can inhibit AD process and lower the potential methane yield of AD reactors (Nielsen & Angelidaki, 2008; Yenigün & Demirel, 2013).

To solve the ammonia problem, many solutions have been proposed in recent years. For example, dilution of the reactor content with water (Nielsen & Angelidaki, 2008); air stripping (Zhang et al., 2012); addition of absorbing material (Hansen et al., 1999); lowering the operating temperature (Angelidaki & Ahring, 1994); co-digestion with high carbon content substrate (Tsapekos et al., 2017); microbial electrochemical cell (Zhang & Angelidaki, 2015) and bioaugmentation with syntrophic acetate oxidizing bacteria (SAOB) or methanogens (Fotidis et al., 2014b; Westerholm et al., 2012). However, many of these methods are technically complex connected with high operational costs leading to limited applicability. Acclimation of microbial consortia to high ammonia levels could provide a practical and cost-effective method to digest protein-rich substrates (Yenigün & Demirel, 2013). A large number of investigations has demonstrated that methanogenic inocula have a high ammonia adaptation potential (Koster & Lettinga, 1988; Parkin et al., 1983). Additionally, a recent research showed that fast and efficient acclimatization of anaerobic consortia to high ammonia levels is possible in batch and fed-batch reactors (Tian et al., 2017). However, limited information (if any) is available in literature about successful acclimation using continuous reactors (e.g. continuously stirred tank reactor (CSTR)) at extremely high ammonia levels ($> 7 \text{ g NH}_4^+-\text{N L}^{-1}$). Lack of successfully acclimatizing the process to extremely high ammonia levels could probably be attributed to the washout effect, by which inhibited microorganisms not growing fast enough at a specific retention time

were washed out from the reactor (Tian et al., 2017). Washout is limiting operation at high rates continuous reactors without any microbial support matrix (e.g. granules).

Previous microbial community assessment studies during different acclimation processes to high ammonia levels, have reported controversial results. On one hand, there are studies indicating that the acclimation process to high ammonia levels caused a shift from acetoclastic to hydrogenotrophic methanogenesis (Schnürer & Nordberg, 2008; Werner et al., 2014). On the other hand, *Methanosarcinaceae* spp. associated acetoclastic pathway has been found to be predominant at high ammonia levels (Calli et al., 2005; Karakashev et al., 2005). A more insightful and detailed understanding about the microbiome would be of great importance in further optimization of stable AD process, thus it is important to elucidate the microbial community changes during acclimation of continuous AD process to extremely high ammonia levels.

Therefore, the main aim of the present study was to use, for the first time, CSTR reactors fed with protein-rich microalgae (3rd generation biomass) as the main substrate, to successfully acclimatize methanogenic consortia to extremely high ammonia levels ($> 7 \text{ g NH}_4^+ \text{-N L}^{-1}$) overcoming the microbial washout effect. An additional aim was to reveal the effect of the ammonia acclimation process in the continuous reactors on the AD microbiome dynamics using next generation 16S rRNA gene sequencing.

2 Material and methods

2.1 Inoculum and feedstock

The inoculum derived from a full-scale mesophilic ($37 \pm 1^\circ\text{C}$) biogas plant (Hashøj, Denmark), fed with 70-90% animal manure and 10-30% food industrial organic waste. Two substrates were used in this study: cattle slurry and microalgae *Chlorella vulgaris*.

Cattle slurry was obtained from Hashøj Municipality, Denmark. It was sieved and stored at -21°C until use. Microalgae *C. vulgaris* (>50% protein in dry matter), as a protein-rich substrate, was grown in mineral salt medium (MBBM-2N) in a raceway pond with continuous illumination at 25°C and pretreated according to a previous methodology (Mahdy et al., 2015) after cultivation and harvest. Specifically, a biological catalyst (Protease, Alcalase 2.5, Novozymes, Denmark) was used to hydrolyse microalgae at pH 8. Subsequently, the inactivation of the enzyme was done by heating the hydrolytic broth to 75°C for 30 mins. Then the pretreated microalgal biomass was stored into freezer until use. The basic characteristics of the inoculum and substrates were shown in Table 1. Ammonium chloride (NH₄Cl, Sigma-Aldrich) was used as ammonia source.

2.2 Experimental setup

Two lab-scale mesophilic (37±1°C) CSTR reactors were used in this study (R1 and R2) as duplicate. Each reactor had a 2.3 and 1.8 L total and working volume, respectively, and was equipped with an influent and an effluent bottle, a feeding peristaltic pump, an electrical heating jacket, a water displacement gas meter and two magnetic stirrers. Both reactors were operating with a hydraulic retention time (HRT) of 23 days throughout the experiment. Cattle manure was used during the start-up period of the reactors until a steady state (defined by the variation of the methane yield less than 10% for at least ten consecutive days) occurred (data shown by Mahdy et al. (2017)). The main experiment was divided into four different phases (Table 2). A mixed substrate of cattle slurry and microalgae (20/80 VS/VS) was used throughout the experiment (P1-4). The acclimation strategy was implemented with stepwise increase of TAN concentration using ammonia chloride (0.5 g NH₄⁺-N L⁻¹ each step) up to 10 g

$\text{NH}_4^+ - \text{N L}^{-1}$ both inside the reactor and the feedstock in the influent bottle. The expected and measured TAN levels and the calculated FAN levels for both reactors are depicted in an E-supplement file.

2.3 Analyses

2.3.1 Physicochemical analyses

TS, VS, TKN and TAN were measured according to APHA (2005). The composition of biogas was measured by a gas-chromatograph equipped with a column of $1.1\text{m} \times 3/16$ "Molsieve 137 and $0.7\text{m} \times 1/4$ " chromosorb 108 (MGC 82-12, Mikrolab A/S, Denmark) using hydrogen as a carrier gas. The pH of samples retrieved from the reactor was determined immediately after retrieval in a closed bottle to minimise evaporation of CO_2 , by PHM99 LAB pH meter. A gas-chromatograph (HP 5890 series II) equipped with flame ionization detector and a FFAP fused silica capillary column ($30\text{ m} \times 0.53\text{ mm i.d.}$, film thickness $1.5\text{ }\mu\text{m}$) was used to measure total VFA concentration inside the reactors, while nitrogen was used as carrier gas.

2.3.2 Microbial analyses

Triplicate samples were taken at the end of each phase to identify the microbial dynamics. After an extra cleaning step with Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1) (Sigma-Aldrich), genomic DNA was extracted from the samples according to PowerSoil® DNA Isolation Kit (MO BIO laboratories Inc., Carlsbad, CA USA). PCR amplification using universal primers 515F/806R was conducted on the V4 region of 16S rRNA gene, and next generation sequencing was performed on an Illumina MiSeq platform (Ramaciotti Centre for Genomics, Kensington, Australia).

Raw reads were deposited in Sequence Read Archive (SRA) database (<http://www.ncbi.nlm.nih.gov/sra>) under the project SPR103296, and detailed sample accession numbers were provided in the E-supplement file. The raw sequences were analysed using CLC Workbench software (V.8.0.2) with microbial genomics module plug in (QIAGEN). Trimming step and chimera crossover filter were applied using the default parameters of the software to ensure the high quality of the reads. Operational taxonomic units' (OTUs) phylogenetic assignment was performed with multiple sequence alignment of fixed length trimmed (240 bp) sequences using MUSCLE software (Edgar, 2004) and Greengenes v13_5 database as reference (clustered at 97%). New OTUs were identified when the similarity percentage with database was lower than 80%, with a minimum occurrence of five reads. Maximum likelihood phylogeny was created with neighbour Joining as construction method and Jukes Cantor as nucleotide substitution model, while 100 bootstrap replicates was used to test the certainty of the evolutionary relation and distance. Rarefaction curves were used to evaluate the sequencing depth for each sample, which was presented in the E-supplement file. Chao 1 bias-corrected index was used to evaluate Alpha diversity, while Beta diversity was represented as Principal Component Analysis (PCA) performed by STAMP software. Statistics regarding the sequencing results and taxonomic assignment were reported in the E-supplement file. The taxonomic assignment of the most interesting OTUs (relative abundance higher than 1% for bacteria and 0.1% for archaea) was based on the results from BLASTN search against 16S ribosomal RNA sequences (Bacteria and Archaea) database and CLC software, and details can be found in the E-supplement file. Microbial relative abundance and corresponding folds change were represented as heat maps (Fig. 3) using Multi experiment viewer software (MeV 4.9.0) (Saeed et al., 2006).

2.4 Calculations and statistics

The FAN concentration was calculated by the following equation:

$$\text{FAN} = \frac{\text{TAN}}{1 + \frac{10^{-\text{pH}}}{K_a}} \quad \text{Eq. (1)}$$

Where K_a is the dissociation constant, which equals to 1.29×10^{-9} at mesophilic condition. All statistical analyses and the plotted data of the reactor performance (methane yield, pH and VFA variation) were made using the OriginLab program (OriginLab Corporation, Northampton, Massachusetts). One-way ANOVA was used to evaluate the statistically significant differences ($p < 0.05$) of the reactor performance. STAMP software was used to identify the OTUs with significant changes in relative abundance of different samples (Parks & Beiko, 2010).

3 Results and discussion

3.1 Reactor performance

During P1, an average methane yield of 338 ± 14 and 338 ± 16 NmL $\text{CH}_4 \text{ g}^{-1}$ VS under steady state was observed for R1 and R2, respectively (Fig. 1a), which was used as the baseline to evaluate any instability during the acclimation process (P2-4). The methane production for both reactors was mostly stable throughout the ammonia acclimation period; only R1 had two brief periods (during P3 and P4) with statistically ($p < 0.05$) lower methane production compared to baseline (P1) but in both cases, it recovered in less than half HRT. Thus, an average methane yield of 322 ± 37 and $327 \pm$

17 NmL CH₄ g⁻¹ VS was achieved for R1 and R2, respectively, throughout the acclimation period (P2-4), which were above 95% of the methane yields during the baseline period (P1). By contrast, previous studies have reported inhibition in continuous reactors exposed to ammonia concentrations above 5 g NH₄⁺-N L⁻¹ (Yenigün & Demirel, 2013). Additionally, another modelling study even defined 10 g NH₄⁺-N L⁻¹ as the absolute AD-death threshold (Liu & Sung, 2002). Interestingly, both reactors in the present study had a stable performance at extreme ammonia levels contradicting/challenging this theoretical threshold. To date, only methanogenic cultures cultivated in batch reactors have remained active at extremely high ammonia levels (> 7 g NH₄⁺-N L⁻¹) (Koster & Lettinga, 1988).

The VFA accumulation (Fig. 1b) in R2 was always lower than R1 during the acclimation process. However, both reactors had VFA concentration below 2400 mg HAc L⁻¹ at the highest ammonia levels with stable methane yield. That was in accordance with previous studies where methane production wasn't affected when the VFA reaches 3000 mg HAc L⁻¹ and inhibition was only observed above 4000 mg HAc L⁻¹ (Ahring et al., 1995; Siegert & Banks, 2005). At the same time, pH fluctuated between 8.4 and 7.7 (Fig. 1c), which was within the normal range (6.5–8.5) for AD process.

Therefore, all the reactor performance parameters denote a stable and efficient acclimation process to extremely high ammonia levels. This process stability could be attributed to two basic reasons. First, even though TAN levels were increased 1.6-folds to 10 g NH₄⁺-N L⁻¹, the FAN levels were stable (800-850 mg NH₃-N L⁻¹, E-supplement file) throughout the experiment due to the lower pH along with the ammonia increase (Fig. 1c). This supports the hypothesis expressed by many researchers that FAN is the

major toxic form of ammonia (Massé et al., 2014). Second, it has been reported that trace elements were crucial to the growth of microbes and also required for efficient anaerobic digestion at high ammonia levels; especially for the interspecies electron transfer (Banks et al., 2012). The cultivation medium of *C. vulgaris*, contains sufficient amounts of trace elements, such as Co and Mo (Mendez et al., 2013), which could have promoted the growth of bacteria and archaea under the extreme ammonia levels. Overall, the current study, using protein-rich microalgae as primary substrate, has shown for the first time that a successful continuous AD acclimation process to extremely high ammonia levels is possible.

3.2 Microbial community dynamics

Alpha diversity (Fig. 2a) based on Chao 1 bias-corrected index, decreased alongside with the increase of ammonia levels in both reactors. This indicates that only some of the initial microbiome members survived at higher ammonia toxicity levels and thus a more narrowed and specialized AD community was formed. Beta diversity (Fig. 2b and Fig. 2c) demonstrated a clear microbial dynamic change throughout the ammonia acclimation process. Additionally, the longest and shortest matrix distances were found between P4 and P1, and P2 and P1, respectively, for both reactors. This indicates that higher ammonia levels had bigger community differences compared to the baseline ammonia level (P1). The result was in agreement with a previous study, which declared that ammonia levels significantly affected the microbiome clustering of different biogas plants (De Vrieze et al., 2015).

3.3 Microbiome composition

The vast majority of AD microbial community throughout the whole experiment was composed by bacteria (98% of relative abundance on average). *Proteobacteria* (30%-40%), *Firmicutes* (20%-30%), followed by *Bacteroidetes* and *WWE1* (both 3%-10%), were the most dominant phyla, as can be seen from the E-supplement file, in agreement with previous study (De Vrieze et al., 2015). *Alphaproteobacteria* and *Clostridia* were the most abundant classes, while *Rhizobiales*, *Clostridiales* and the uncultured MBA08 were predominant at order level.

The first and second most abundant bacterial OTUs (an average abundance of 22.09% and 6.50%, respectively) belonged to *Rhizobiaceae* family. However, both OTUs showed no significant change of relative abundance during this acclimation process (Fig. 3). By performing a BLASTN search, the best matches for the two OTUs were identified with 100% identity to *Shinella* spp. and *Rhizobium kunmingense*, respectively. *Shinella* spp. was previously identified in a biogas reactor treating grass silage and lignocellulose substrate (Wang et al., 2010), and also found, with high relative abundance, in reactors with pure microalgae as substrate (Sanz et al., 2017). Thus, it was proposed by Sanz et al. (2017) that *Shinella* spp., may play a pivotal role in microalgae degradation, especially for some recalcitrant polysaccharides and glycoprotein matrix. The exact function of *Shinella* spp. during AD process is still unclear; however, the results of the current study indicate that its high abundance was not affected by the ammonia levels. Regarding the second abundant OTU, it seems that *R. kunmingense* was delivered into the reactors together with the microalgae feedstock (*C. vulgaris*). This is supported firstly by Wirth et al. (2015) who reported the presence of *R. kunmingense* in a two-stage biohydrogen and biogas reactor fed only with *Chlamydomonas* spp. and *Scenedesmus* spp. algae; and secondly by Kim et al. (2014)

who found relative abundance of *Rhizobium* spp. was as high as 30.23% during *C. vulgaris* cultivation.

Among the highly abundant bacteria, 27 and 25 OTUs for R1 and R2, respectively, changed significantly from P1 to P4 (Fig. 4), indicating a sensibility of these microbes in respect to ammonia. Regarding microbes that seem favoured, 4 *Clostridia*, *Wohlfahrtiimonas* sp. 11 and *Cloacamonae* sp. 26 were the OTUs that significantly increased in relative abundance. Specifically, *Clostridium* sp. 13 was the most interesting bacterium, increasing by 37 and 60 folds (0.1% in P1 to 3.7%-6.0% in P4, Fig. 3) for R1 and R2, respectively, and was found to be 95% similar to an isolated SAOB *Clostridium ultunense* (Schnürer, 1996). This result was in accordance with previous studies (Westerholm et al., 2011), which found *C. ultunense* increased significantly under elevated ammonia levels. It is known that SAOB are always coupled with hydrogen utilization methanogens (Hattori, 2008), thus the increased abundance of *C. ultunense* indicated a significant hydrogenotrophic methanogenic activity. Another noteworthy result was that order MBA08 (consisted of *Clostridia* sp. 5, *Clostridia* sp. 7, *Clostridia* sp. 10 and *Clostridia* sp. 16), increased from 9.3% to 12.5%. MBA08 was found in mesophilic AD processes with increased ammonia levels (Müller et al., 2016), and also in biogas upgrading reactors operating under a dominant hydrogenotrophic pathway (Kougias et al., 2017). Based on BLASTN search result, these OTUs showed 88-91% identity to *Hydrogenispora ethanolica*, a carbohydrate-fermenting bacterium with the ability to produce hydrogen (Peng et al., 2014). Even though taxonomical and functional classification of this microbe needs further investigation, it still can be concluded that *H. ethanolica* can tolerate high ammonia levels. Regarding the OTU negatively influenced by ammonia, the most inhibited *Cloacamonae* sp. 2 was affiliated

to WWE1 group, which was found in manure-based biogas reactors, and was assumed to participate in the cellulose catabolic processes (Limam et al., 2014). Thus, high abundance of *Cloacamonae* sp. 2 at P1 could be explained by the manure-based inoculum and the cattle manure used to start-up the reactors. The significant decrease in abundance was most probably caused by low ammonia tolerance.

Considering the archaeal community, *Methanosarcina* sp. 1 and *Methanobrevibacter* sp. 2 were the predominant methanogens at P1, with more than 30% and 60% of the archaea relative abundance, respectively (0.45% and 0.8% of the total microbial community). However, at the highest ammonia level (P4), the two OTUs showed opposite behaviours: the relative abundance of *Methanobrevibacter* sp. 2 collapsed to less than 1%, while *Methanosarcina* sp. 1 became the most abundant methanogen (almost 90%). Moreover, *Methanoculleus palmolei* 3 emerged in P4 and became the second most abundant archaea (5-8%). This impressive positive change of *Methanosarcina* spp. abundance contrasted with previous studies, which reported that hydrogenotrophic methanogens (i.e. *Methanomicrobiales* spp. and *Methanobacteriales* spp.) were predominant at high ammonia levels (Angenent et al., 2002; Fotidis et al., 2014a). However, it has been reported by Jarrell et al. (1987) that *Methanosarcina barkeri* can tolerate extremely high ammonia levels ($10 \text{ NH}_4^+ \text{-N L}^{-1}$). In this study, based on BLASTN analysis, *Methanosarcina* sp. 1 was found to be equally similar to different *Methanosarcina* species (*M. soligelidi*/ *acetivorans*/ *barkeri*/ *mazei*) with 98% identity, thus is not possible to identify it at species level. Anyway, *Methanosarcina* sp. 1 ammonia tolerance could be explained by the known specific morphology of *Methanosarcina* genus; In detail, its large cell size and spherical shape was reported to form large multicellular structure (clusters) with higher volume/ surface ratio at high

ammonia levels (Calli et al., 2005; Goberna et al., 2010). More over organisation in clusters could create a niche environment with lower ammonia concentrations due to ammonia concentration gradient from the surface of the cluster to the inner core. Another explanation could be the reported ability of some *Methanosarcina* species to shift their metabolic pathway into hydrogenotrophic methanogenesis under extreme conditions (e.g. high ammonia levels) (Demirel & Scherer, 2008; Thauer et al., 2008). Since SAOB abundance increased significantly alongside with *Methanosarcina* spp. during this acclimation process (Fig. 3), there is the possibility that some of the *Methanosarcina* species were mediating the hydrogenotrophic pathway. However, assessing the methanogenic pathway of *Methanosarcina* spp. was out of the scope of the current study. Finally, *Methanoculleus palmolei* 3 increased in abundance, which was in agreement with a previous study where *Methanoculleus* spp. increased proportionally with the ammonia levels (Westerholm et al., 2012). However, not all hydrogenotrophic methanogens were able to cope with ammonia toxicity. In fact, the abundance of *Methanobrevibacter* sp. 2 decreased significantly during the acclimation process, indicating its limitation to thrive at extremely high ammonia levels.

4 Conclusions

The stepwise acclimation strategy used in the present study showcases the potential of utilizing protein-rich microalgae (3rd generation biomass) as AD substrates and paves the way forward to use other protein-rich substrates (e.g. food waste, slaughterhouse waste etc.). Moreover, 16s rRNA sequencing showed a clear shift in the microbial dynamics throughout the acclimation process, which led to a narrower and specialized (ammonia tolerant) AD community. Surprisingly, facultative acetoclastic *Methanosarcina* spp. was the most dominant methanogen at extreme ammonia levels.

This indicates that there are acetoclastic methanogens capable to thrive even under very hostile conditions (i.e. washout effect, extreme ammonia levels).

Appendix A. Supplementary material

E-supplementary data for this work can be found in e-version of this paper online.

Supplementary data 1: Fig S1. The estimated and measured TAN levels and calculated FAN concentration throughout the experimental period; Fig S2. Rarefaction curve of all microbial samples; Table S1. Sample name and the corresponding accession number in SRA database; Table S2. Statistics of the sequencing process and data analysis of the 16S rRNA samples.

Supplementary data 2: Table S3. Taxonomic assignment of 16S rRNA gene of interesting OTUs at different phases in R1 and R2.

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Highlights

- Successful acclimation of methanogenic culture to extreme ammonia levels in CSTR
- Efficient utilization of a 3rd generation biomass as biomethanation substrate
- The most abundant bacterium (*Shinella* spp.) was not affected by the ammonia levels
- *C. ultunense* increased significantly its abundance during the acclimation process
- *Methanosarcina* spp. was the most abundant methanogen at the highest ammonia levels

Table 1. Characteristics of the inoculum and substrates.

Parameter	Inoculum	Cattle slurry	Microalgae
	value $\pm$ SD ^a	value $\pm$ SD ^a	value $\pm$ SD ^a
Total solids-TS (g L ⁻¹)	33.20 $\pm$ 0.19	32.90 $\pm$ 0.02	160.00 $\pm$ 0.24
Volatile solids-VS (g L ⁻¹)	19.80 $\pm$ 0.18	23.00 $\pm$ 0.04	138.66 $\pm$ 0.18
Total Ammonium nitrogen-TAN (g NH ₄ ⁺ -N L ⁻¹)	4.58 $\pm$ 0.02	1.10 $\pm$ 0.12	3.44 $\pm$ 0.36
Total Kjeldahl nitrogen-TKN (g N L ⁻¹)	5.01 $\pm$ 0.13	1.49 $\pm$ 0.01	14.29 $\pm$ 0.18
Volatile fatty acids-VFA (mg L ⁻¹)	76.08 $\pm$ 5.75	8936.97 $\pm$ 50.51	2668.94 $\pm$ 68.91

^a Standard deviation**Table 2.** Operational parameters in different experimental phases.

	Phase 1	Phase 2	Phase 3	Phase 4
	(P1)*	(P2)	(P3)	(P4)
Days	0-28	29-51	52-98	99-133
Substrate	20% cattle slurry + 80% microalgae (based on VS)			
Organic loading rate-OLR (g VS L ⁻¹ d ⁻¹)	1.95 $\pm$ 0.1			
Total ammonium nitrogen-TAN (g	3.3-3.8	4.3-6.0	6.5-8.0	8.5-10

$\text{NH}_4^+ \cdot \text{N L}^{-1}$)

*P1 was used as the baseline before acclimation strategy with addition of NH_4Cl .

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Figure Captions

Fig. 1. The performance parameters of the reactors a) methane yield, b) VFA and c) pH at different experimental phases. Arrows indicate the sampling time for microbial analysis.

Fig. 2. Alpha Diversity (a) based on Chao 1 bias-corrected index; Beta diversity of the triplicate samples in R1 (b) and R2 (c). Principal components (PC) 1 and 2 explained 62.3% and 21.6% of community variation for R1, and 65.5% and 19.5% for R2, respectively.

Fig. 3. Relative abundance (%) (left part) and the corresponding folds change (right part) for the interesting archaea in reactor R1 (a) and R2 (b), and bacteria in reactor R1 (c) and R2 (d).

Fig. 4. OTUs that changed significantly ($p < 0.05$) in R1 (a) and R2 (b) in the comparison between P4 and P1.