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

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Abstract

Different mesophilic and thermophilic methanogenic consortia were acclimatised and enriched to extreme total ammonia (9.0 and 5.0 g $\text{NH}_4^+\text{-N L}^{-1}$, respectively) and free ammonia (1.0 and 1.4 g $\text{NH}_3\text{-N L}^{-1}$, respectively) levels in this study. $[2\text{-}^{14}\text{C}]$ acetate radioisotopic analyses showed the dominance of acetoclastic methanogenesis in all enriched consortia. According to 16S rRNA gene sequencing result, in mesophilic consortia, methylotrophic *Methanomassiliicoccus luminyensis* was predominant, followed by acetoclastic *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, acetoclastic *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.

Keywords

Anaerobic digestion; Enriched culture; Methanogenic pathway; Ammonia-tolerant microbial community; *Methanomassiliicoccus luminyensis*.

1 Introduction

Anaerobic digestion (AD) is a widely used biotechnological process to treat a variety of biowaste and recover renewable energy (CH₄). 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 methanogenesis (Eq.(2-3)) (Zinder and Koch, 1984). The former pathway is mainly performed by *Methanosaetaceae* and *Methanosarcinaceae*, while the latter one by *Methanococcales*, *Methanobacteriales*, *Methanocellales* and *Methanomicrobiales* (Lyu and Lu, 2015).

In recent years, several nitrogen-rich biowastes (e.g. poultry manure, pig manure, slaughterhouse waste, etc.) have been considered as feedstock for biogas production due to their high methane potential. However, ammonia, which is a major toxicant of the AD process, is also produced during anaerobic degradation of nitrogen-rich biowaste (Fotidis et al., 2017). Methanogenesis is generally considered to be the most vulnerable AD process step to ammonia toxicity (Yenigün and Demirel, 2013). Chen et al. (2008) reviewed that total ammonia (TAN = NH₄⁺ + NH₃) concentrations ranging from 1.7 to

14.0 g $\text{NH}_4^+\text{-N L}^{-1}$ could cause 50% loss of the methane potential, depending on the operational conditions. Additionally, free ammonia (FAN, NH_3) levels, which depend on temperature, pH and TAN levels, were reported to be the most toxic form of ammonia due to intracellular interference on biological activities of the methanogens (Massé et al., 2014).

Recently, the use of ammonia-tolerant methanogenic consortia was proposed as a potential solution to alleviate the ammonia toxicity problem in anaerobic reactors (Hao et al., 2015). Enrichment of ammonia-tolerant consortia, at both mesophilic and thermophilic conditions, is of great interest to optimize the utilization of nitrogen-rich substrates. Some researchers have already acclimatized different methanogenic cultures to high ammonia levels under mesophilic and thermophilic conditions (Tian et al., 2017). However, the microbiological characteristics of such consortia are still not clear. For example, it was reported (Sun et al., 2014; Müller et al., 2016) that hydrogenotrophic methanogens became dominant at high ammonia levels ($>3.0 \text{ g NH}_4^+\text{-N L}^{-1}$) with a simultaneous increase of the relative abundance of the syntrophic acetate oxidising bacteria (SAOB). Moreover, *Methanomicrobiales* and/or *Methanobacteriales* were detected to be predominant at TAN levels higher than 2.8 and up to 19 g $\text{NH}_4^+\text{-N L}^{-1}$ (Poirier et al., 2017; Ruiz-Sánchez et al., 2018). However, many researchers have presented controversial observations. For example, a member of aceticlastic *Methanosarcinaceae* was found to be the dominant methanogen in a mesophilic culture that was stepwise acclimatized in batch reactors above 5.0 g $\text{NH}_4^+\text{-N L}^{-1}$ (Fotidis et al., 2013). Furthermore, Tian et al. (2018b) found that *Methanosarcina* spp. were the most abundant archaea in continuous reactors at FAN levels above 750 mg $\text{NH}_3\text{-N L}^{-1}$. Considering these contradictions, further investigation on the ammonia-tolerant

methanogenic consortia is necessary. Moreover, besides methanogens, SAOB were also reported to play an important role on catabolizing acetate under high ammonia stress. Up to date, only three mesophilic SAOB (i.e. *Clostridium ultunense* (Schnürer, 1996), *Syntrophaceticus schinkii* (Westerholm et al., 2010) and *Tepidanaerobacter acetatoxydans* (Westerholm et al., 2011)) and two thermophilic SAOB (i.e. *Thermacetogenium phaeum* (Hattori et al., 2000) and *Pseudothermotoga lettingae* (Balk et al., 2002)) have been isolated. Some microbes without isolation, belonging to genus *Coprothermobacter*, orders *Bacteroidales*, *Clostridiales* and *Thermoanaerobacterales*, and phylum *Thermotogae* were also proposed to perform SAO (Nobu et al., 2015; Müller et al., 2016). Thus, a comprehensive and deep insight into the whole biomethanation community (archaea and bacteria) of ammonia-tolerant consortia is of great relevance to facilitate the way of developing and utilizing the microbiota to overcome ammonia inhibition. This knowledge will allow applying efficiently new biological methods (e.g. bioaugmentation technology), based on ammonia-tolerant consortia, to overcome the ammonia toxicity effect (Fotidis et al., 2014b).

Although the microbial community composition gives a good insight on the ammonia-tolerant consortia, the dominant methanogenic pathway should also be identified. This will allow to assess the importance of different methanogens and especially those that can perform both acetoclastic and hydrogenotrophic pathway, such as *Methanosarcina* species (De Vrieze et al., 2012). Radioisotopic analyses with labelled methyl group [2-¹⁴C] of sodium acetate can be used to identify the dominant acetate methanogenic pathway (Schnürer and Nordberg, 2008; Fotidis et al., 2013). Thus, it is a great opportunity to combine the radioisotopic analysis with the advanced

microbial sequencing technology to understand fully the microbial characteristics of the ammonia-tolerant methanogenic consortia.

Therefore, the main aims were first, to enrich different methanogenic cultures adapted to high ammonia levels at both mesophilic and thermophilic conditions; second, to apply a combination of 16S rRNA gene sequencing and radioisotopic acetate pathway analysis to characterize the ammonia-tolerant methanogenic consortia. To fulfil these aims, two mesophilic and three thermophilic inocula from different full-scale biogas plants, were enriched with stepwise increased ammonia levels in batch reactors. Furthermore, the final ammonia-tolerant consortia were characterized by deciphering the microbial composition and identifying the dominant acetate methanogenic pathway.

2 Material and methods

2.1 Initial inocula

Two mesophilic (Fangel (M1) and Hashøj (M2)) and three thermophilic (Blåhøj (T1), Ribe (T2) and Snertinge (T3)) initial inocula were used in this study. All inocula were derived from Danish full-scale biogas plants. All the reactors were manure-based continuously stirred tank reactors (CSTR) with cattle and pig manure as main feedstocks, while different biomasses were used as secondary feedstocks (e.g. poultry manure, mink slurry, food industry waste, medicinal biowaste etc.). The detailed characteristics of the used inocula and the operational parameters of the five full-scale biogas plants are shown in Table 1.

2.2 Enrichment procedure

Batch reactors with 40 and 118 mL working and total volume, respectively, were used to enrich the ammonia-tolerant consortia. Basal anaerobic medium (BA medium (E-supplementary data)), containing the basic nutrients and microelements for microbial growth (Angelidaki et al., 1990), was used as cultivation medium throughout the experiment. The inoculum/working volume ratio was set as 12.5% (v/v). The whole enrichment processes were divided into seven steps (the details experimental setup is depicted in Table 2). During steps 1 to 5, mixed N₂/CO₂ (80/20% v/v) gas was used to flush the reactors' content, to create anoxic conditions and keep the pH at 7.0±0.1. Meanwhile, all enrichment batches were started from TAN levels of 3.0 g NH₄⁺-N L⁻¹, and then stepwise (1.0 g NH₄⁺-N L⁻¹ each step) increased to 7.0 g NH₄⁺-N L⁻¹. At step 6 and 7, the pH of the reactors was increased to 8.0±0.1 by flushing with pure N₂ gas and adding NaOH solution (when necessary) to create a high FAN environment. Moreover, for mesophilic inocula, TAN levels increased from 7.0 g NH₄⁺-N L⁻¹ (Step 6) to 9.0 g NH₄⁺-N L⁻¹ (Step 7). At the same time, for the thermophilic inocula, the TAN levels were decreased from 7.0 to 5.0 g NH₄⁺-N L⁻¹ (Step 6), due to the already high FAN levels (1.4 g NH₃-N L⁻¹) at 5.0 g NH₄⁺-N L⁻¹.

Throughout the enrichment process, Na₂S·9H₂O (final concentration: 62.5 mg L⁻¹) and yeast extract (final concentration: 0.2 g L⁻¹) were added to all the bottles to ensure anaerobic conditions and supplement of necessary vitamins, amino acids and other growth factors, respectively. Furthermore, acetate (2 g L⁻¹ during all the enrichment steps except for the last step, which 3 g L⁻¹ were used to get a high biomass concentration for microbial analyses) was used as carbon source, while ammonium chloride was chosen as ammonia source. After closing with butyl rubber stoppers and sealing with aluminium caps, all the bottles were incubated in triplicates at their

corresponding temperatures. The acclimatization at each step was stopped when the stationary phase was achieved and the methane production was at least 90% of the maximum theoretical expected production.

2.3 Radioisotopic experimental setup

A radioisotopic test using labelled [2- ^{14}C] sodium acetate (Amersham Pharmacia Biotech, UK) was performed on the final enriched consortia, to identify the dominant acetate methanogenic pathway. To follow the methanogenic pathway, each enriched consortium was mixed with BA medium and introduced to batch reactors with three different setups: 1) blanks, to evaluate the background methane production; 2) control, with unlabelled acetate (1.0 g L^{-1}), to follow methane production; 3) labelled bottles, with both labelled ($54.90 \pm 0.46 \text{ KBq L}^{-1}$) and unlabelled (1.0 g L^{-1}) acetate, to evaluate the $^{14}\text{CO}_2/^{14}\text{CH}_4$ ratio. The ^{14}C radioisotopic activity was measured by a liquid scintillation counter (Hidex 300 SL; LabLogic Company, United Kingdom), and the samples were prepared according to Fotidis et al. (2013). To release the dissolved $^{14}\text{CO}_2$ in the reactors with [2- ^{14}C] acetate addition, 2 mL 7.2M HCl solution was added into each reactor to acidify the solution when methane production ceased. The produced $^{14}\text{CO}_2$ was trapped with CO_2 absorber (Carbosorb^R-E; PerkinElmer Company). The produced $^{14}\text{CH}_4$ was oxidized to $^{14}\text{CO}_2$ in a tube furnace (at 850°C) with copper oxide (CuO, Sigma-Aldrich) as catalyst, and trapped with a different aliquot of the same CO_2 absorber. Meanwhile, the liquid sample from the reactors was mixed with distilled water to identify the residual [2- ^{14}C] acetate radioactivity. Finally, all the samples were mixed with scintillation liquid (Permafluor E⁺ for $^{14}\text{CO}_2$ trapped samples and Optiphase Hisafe 3 for reactor liquid samples; PerkinElmer Company) before measuring with the scintillation counter.

2.4 Microbial analyses with amplicon sequencing

To elucidate the microbial community composition, 90 mL samples of the enriched consortia were taken from the last step of the enrichment process (i.e. step 7 for mesophilic and step 6 for thermophilic enriched cultures). PowerSoil® DNA Isolation Kit (MO BIO laboratories Inc., Carlsbad, CA USA) was used for the genomic DNA extraction. Furthermore, an extra cleaning step with Phenol: Chloroform: Isoamyl Alcohol (25: 24: 1) (Sigma-Aldrich) was performed to guarantee the DNA quality. The quantity and quality of the extracted DNA were determined by NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA). PCR amplification on V4 region of 16S rRNA gene was conducted using 515F/806R universal primers, and Illumina MiSeq platform (Ramaciotti Centre for Genomics, Kensington, Australia) was used to generate paired-end sequencing reads (2×250bp). CLC Workbench software (V.8.0.2) with microbial genomics module plugin was used to analyse the raw sequence data for quality filtering, diversity analyses, OTUs (operational taxonomic units) clustering and taxonomy identification (Greengenes v13_5 database). The adequate sequencing depth for all samples was ensured by rarefaction curves (E-supplementary data). Principal Coordinate Analysis (PCoA) was used to estimate beta diversity. BLASTN search against NCBI (National Center for Biotechnology Information) 16S ribosomal RNA sequences (Bacteria and Archaea) database was performed on the interesting (relative abundance higher than 0.5%, provided by CLC software) OTUs' consensus sequences to verify the taxonomic assignment (E-supplementary data). Furthermore, relative abundance of the interesting OTUs was presented as heat maps using Multi experiment viewer software (MeV 4.9.0) (Saeed et al., 2006). The raw sequencing reads were

deposited in Sequence Read Archive (SRA) database (<http://www.ncbi.nlm.nih.gov/sra>) under the ID PRJNA475596.

2.5 Physicochemical analyses

TS, VS, and TAN were measured according to APHA (2012). The pH was measured by PHM99 LAB pH meter. The methane production was determined by a gas chromatograph (Trace 1310 GC-TCD, Thermo Fisher, Denmark) equipped with a thermal conductivity detector. A TracePLOT TG-BOND Q 26004-6030 column (30 m × 0.32 mm I.D., film thickness 10 µm) (Thermo Fisher) was used, and the column flow rate was 2.7 mL min⁻¹. The temperature of the front inlet, oven and detector were 150°C, 70°C and 200°C, respectively. Helium was used as carrier gas. The VFA levels were measured through a gas-chromatograph (Shimadzu GC-2010, Kyoto, Japan) equipped with flame ionization detector and a FFAP fused silica capillary column (30 m × 0.53 mm i.d., film thickness 1.5 µm). The temperature of the injection port and the detector were 150°C and 230°C, respectively. Nitrogen was used as carrier gas.

2.6 Calculations and statistics

OriginLab (OriginLab Corporation, Northampton, Massachusetts) program was used to perform all the statistical analyses and to prepare the figures.

2.6.1 Free ammonia

The FAN levels were calculated by the following equation:

$$\text{FAN} = \frac{\text{TAN}}{1 + \frac{10^{-\text{pH}}}{K_a}} \quad \text{Eq. (4)}$$

Where K_a is the dissociation constant determined by temperature and it equals to 1.29×10^{-9} at mesophilic condition ($37 \pm 1^\circ\text{C}$) and 3.91×10^{-9} at thermophilic condition ($55 \pm 1^\circ\text{C}$).

2.6.2 Acetate methanogenic pathway

The ratio of $^{14}\text{CO}_2/^{14}\text{CH}_4$ from the radioisotopic analyses was used to estimate the dominant acetate methanogenic pathway. It is generally accepted that the dominant acetate methanogenic pathway is aceticlastic when $^{14}\text{CO}_2/^{14}\text{CH}_4 < 1$, and SAO coupled with hydrogenotrophic methanogens is dominant when $^{14}\text{CO}_2/^{14}\text{CH}_4 > 1$ (Fotidis et al., 2013).

2.6.3 Maximum specific growth rate

The OriginLab software was used to calculate the maximum specific growth rate ($\mu_{\max}$) of the methanogenic consortia according to the slope of the linear part of the semi-logarithmic graph of the methane production versus the incubation time (Gray et al., 2009).

3 Results and discussion

3.1 Enriched cultures

In four out of five enriched methanogenic consortia (except M1), the $\mu_{\max}$ decreased with the increase of ammonia levels during the enrichment process (Fig. 1), which agreed with previous observations of decreased growth rates at high ammonia levels (Yenigün and Demirel, 2013). The maximum $\mu_{\max}$ for each one of the cultures was observed during step 1 of the enrichment process (i.e. at the lowest ammonia levels), with the exception of culture M1 where the highest $\mu_{\max}$ was observed during steps 2 and 3 (no statistical difference, $p > 0.05$). Ranging from 0.47 to 0.79 d^{-1} , the maximum

$\mu_{\max}$ of all the enriched cultures in this study was comparable with other studies. For example, Fotidis et al. (2013) observed $\mu_{\max}$ of acetate-fed methanogenic cultures varying from 0.3 to 0.7 d⁻¹ at TAN levels between 2.0 and 5.0 g NH₄⁺-N L⁻¹ for both mesophilic and thermophilic conditions. Moreover, the $\mu_{\max}$ of pure strains (*Methanosarcina thermophila* and *Methanoculleus thermophilus*) was also reported to be within 0.5 and 1.0 d⁻¹ at FAN levels from 0.1 up to 1.4 g NH₃-N L⁻¹ (Tian et al., 2018a). However, the $\mu_{\max}$ of the enriched cultures, were significantly lower than the $\mu_{\max}$ measured in biomethanation microbiomes of several full-scale digesters (1.2 to 1.5 d⁻¹) (Fotidis et al., 2014a). This could be explained by higher FAN levels (0.39-0.86 g NH₃-N L⁻¹) to which the enriched consortia were exposed, compared to the aforementioned study (0.05-0.51 g NH₃-N L⁻¹).

Even though the enrichment process ended with a certain degree of $\mu_{\max}$ reduction (Fig. 1), the results showed that the enriched ammonia-tolerant consortia were still able to work efficiently at high ammonia levels (i.e. TAN levels of 9.0 and 5.0 g NH₄⁺-N L⁻¹, and FAN levels of 1.0 and 1.4 g NH₃-N L⁻¹, under mesophilic and thermophilic conditions, respectively). The $\mu_{\max}$ of the final ammonia-tolerant consortia varied from 0.21 to 0.31 d⁻¹ for all the samples, except for T3, which was significantly ($p < 0.05$) lower (0.17 d⁻¹) than the others. However, efficient anaerobic degradation of poultry manure was possible with an overall $\mu_{\max}$ around 0.1 d⁻¹ (Hunik et al., 1990), which indicates that the enriched cultures in the current study have adequate growth rates to mediate the digestion of ammonia-rich substrates in CSTR biogas reactors, considering that commonly used HRTs are in the range 13-25 days. Ammonia-tolerant microbial consortia with growth rates corresponding to doubling times of 2-3 days would stay in reactor and outcompete ammonia inhibited microbes at high ammonia loadings.

3.2 Methanogenic pathway

The acetoclastic pathway ($^{14}\text{CO}_2/^{14}\text{CH}_4 < 1$) was identified to be the dominant acetate methanogenic pathway in all the ammonia-tolerant consortia (Fig. 2). This coincides with previous researches, where acetoclastic methanogenesis became dominant when TAN levels increased from 0.26 to 7.0 g $\text{NH}_4^+\text{-N L}^{-1}$ (Fotidis et al., 2013). Moreover, based on stable carbon (1, 2- ^{13}C) isotopic analysis, acetate was identified to be catabolised to methane through the acetoclastic pathway, regardless of ammonia concentration (Hao et al., 2015). On contrary, other researchers have found that the methanogenic pathway changed from acetoclastic to hydrogenotrophic when ammonia levels increased (Schnürer and Nordberg, 2008). This difference of the dominant acetate methanogenic pathway at high ammonia levels might be due to the different operational conditions (e.g. various substrates, different HRTs, etc.) of the biogas plant, where the initial inocula derived from.

3.3 Global microbial diversity

In total, more than six million raw sequences were generated from the triplicate samples of the five ammonia-tolerant consortia. Beta diversity was expressed based on PCoA (Fig. 3), with PCo1 and PCo2 explaining 81% and 8% variation in diversity, respectively. Additionally, the PCoA showed a big difference between mesophilic and thermophilic consortia, which was expected because temperature is a crucial factor influencing the microbial composition (Treu et al., 2018). However, among the three thermophilic ammonia-tolerant consortia, T1 and T2 shared similarity, but they were different from T3. This difference might be contribute to the lower μ_{max} that T3 had at the last acclimation step compared to T1 and T2 (Fig. 1), which indicated that the final microbiome compositions could be shaped differently by the ammonia toxicity.

3.4 Microbial community composition

In mesophilic consortia, relative abundance of archaea reached approximately 30% and the other 70% was bacteria, while in thermophilic consortia, archaea varied between 19% and 24% (E-supplementary data). The abundance of archaea (methanogens) in these consortia was significantly higher compared to many previous studies investigating the microbial composition in AD reactors (Fitamo et al., 2017; Zhu et al., 2017), indicating that the enrichment process with single and simple substrate aiming to obtain high abundance of ammonia-tolerant methanogens was successful.

3.4.1 Mesophilic enriched consortia

At mesophilic conditions, only three orders were found among methanogens: E2, *Methanosarcinales* and *Methanomicrobiales* (Fig. 4a). In these three orders, only three genera were found (Fig. 4b): *Methanomassiliicoccus* spp. (relative abundance of 51% in M1 and 65% in M2 within archaea community), *Methanosarcina* spp. (47% in M1 and 34% in M2) and *Methanoculleus* sp. (2% in M1 and 1% in M2). By conducting a BLASTN search, the two OTUs (Fig. 5a) identified as *Methanomassiliicoccus* spp. were assigned to *Methanomassiliicoccus luminyensis* with 96-98% similarity. *M. luminyensis* was a newly reported methanogen, isolated from human faeces and recently found in biogas reactors (Popp et al., 2016; Lin et al., 2017). Furthermore, *M. luminyensis* was found to be abundant at TAN levels around 6.0-7.0 g NH₄⁺-N L⁻¹ in lab and full scale reactors fed with protein-rich substrates (Ács et al., 2013; Ruiz-Sánchez et al., 2018), which indicates its high tolerance to ammonia toxicity. The predominance of *M. luminyensis* was a surprise, since the dominant acetate methanogenic pathways of M1 and M2 were found to be aceticlastic (Fig. 2). *M. luminyensis* cannot use acetate to produce methane but only methanol, tri-, di- and monomethylamine (strict

methylophilic) with the presence of hydrogen as the electron donor (Dridi et al., 2012). A possible explanation could be that all or some of these specific substrates (i.e. methanol, tri-, di- and monomethylamine), were produced from the degradation of amino acids (derived from yeast extract included in the medium) and/or the cells lysis of species that could not adapt to high ammonia levels (Harms et al., 1998; Dorokhov et al., 2015). This was also supported by the fact that several bacterial OTUs were assigned to genus *Tissierella* and this genus can produce methylamine from different amino acids (Farrow et al., 1995; Harms et al., 1998). Moreover, without having the ability to use acetate to produce methane, *Methanomassiliicoccus* spp. have the genes to use acetate to produce energy for building biomass (KEGG-Database, 2018), which could be an additional explanation for the high abundance of *M. luminyensis*. Last but not least, it was reported that acetate can be reduced to methanol by some bacteria, such as *Shewanella putrefaciens* (Vajda et al., 2011). Even though this specific bacterium was not detected, there might be some uncultured OTUs that can perform this metabolic pathway. Unfortunately, 16s rRNA sequencing analysis only provides information about taxonomic assignment, but not about genomic characteristics and the functional annotation. Moreover, the sequencing length (251bp) of 16s rRNA amplicon sequencing technology also limited the detailed analysis on species level. Therefore, on the one hand, further investigations based on advanced techniques like total random sequences could be performed to further elucidate the metabolic pathway of *Methanomassiliicoccus* spp. detected in this study. On the other hand, isolation of the *Methanomassiliicoccus* spp. could also be done with methylated one-carbon compounds, and then assesses their metabolic pathway. However, the detailed metabolic study of

one specific species is beyond the scope of the current study, thus not being performed and analysed in this paper.

The second most abundant methanogen was found to be *Methanosarcina soligelidi* with a similarity of 98%. The abundance of *M. soligelidi* at high ammonia levels was in accordance to previous study reporting that *Methanosarcina* sp. was dominant in continuous reactors at TAN levels up to 10.0 g NH₄⁺-N L⁻¹ (Tian et al., 2018b). The methanogenic pathway in the aforementioned research was not identified thus a possible hydrogenotrophic pathway was proposed. However, the radioisotopic analysis in the present study indicated that *Methanosarcina* spp. still performed acetoclastic pathway at such high ammonia levels.

Bacterial phyla *Bacteroidetes*, *Firmicutes* and *Synergistetes* constituted more than 90% of the total community (Fig. 4c). However, for almost half of the bacterial OTUs, they were not possible to reach taxonomic assignment at genus level (Fig. 4d). The most abundant OTU (relative abundance of 38% in M1 and 25% in M2 with respect to bacterial community) was represented by *Tepidimicrobium* sp. (Fig. 5a), which can be assigned to *Tepidimicrobium xylanilyticum*. *T. xylanilyticum* is an anaerobic xylanolytic bacterium that uses a variety of carbohydrates and proteinaceous compounds (Niu et al., 2009), thus its high abundance could be attributed to the degradation of yeast extract and dead cells under high ammonia conditions. Similarly, two other abundant OTUs, also able to catabolize amino acids, were identified to be 100% similar to *Fermentimonas caenicola* and *Aminobacterium colombiense*, respectively. Specifically, it has been reported that *F. caenicola* grows on yeast extract, producing mainly acetate, CO₂ and H₂ (Hahnke et al., 2016); additionally, *A. colombiense* is an amino acid

degrading bacterium (Baena et al., 1998), and yeast extract is mainly composed by different types of amino acids .

Four very interesting OTUs, with total relative abundances of 6% and 15% in M1 and M2 with respect to bacteria community, respectively, were assigned to genus *Tissierella*, which was reported to produce methylamine from different amino acids (Farrow et al., 1995; Harms et al., 1998). Thus, it seems that the presence of *Tissierella* spp. was the main reason for the high abundance of the strict methylotrophic *M. luminyensis* discussed above. However, it is not possible to claim that only methylamine (and not acetate) was the sole carbon source that *M. luminyensis* was metabolizing, because metabolomics analysis was not part of the present study.

Two more OTUs that had relative abundance around 1-2%, were assigned with a 98% similarity to *Clostridium ultunense*, which is a well-known SAOB in mesophilic biogas reactors (Schnürer, 1996). This low abundance of SAOB was correlated with the low abundant hydrogenotrophic *Methanoculleus* sp. (Fig. 5a), indicating a certain degree of hydrogenotrophic methanogenesis even with aceticlastic pathway as the dominant acetate metabolic pathway. However, it is also possible that hydrogen derived from the SAOB was used by the *M. luminyensis* as electron donor to produce CH₄ from methanol, tri-, di- and monomethylamine (Dridi et al., 2012).

3.4.2 Thermophilic enriched consortia

Methanosarcinales, *Methanomicrobiales*, *Methanobacteriales* and E2 orders composed of the methanogens of all thermophilic enriched cultures (Fig. 4a). The most abundant genus was *Methanosarcina* (Fig. 4b) and the most abundant OTU was identified to be 100% similar to *Methanosarcina thermophila* (Fig. 5b), a well-known aceticlastic methanogenic species (Zinder et al., 1985). The high relative abundance of

M. thermophila, varying between 65% and 95% within the archaea community in all enriched cultures, was consistent with the dominant aceticlastic methanogenic pathway found in radioisotopic analysis (Fig. 2). The other two abundant OTUs were 100% similar to hydrogenotrophic *Methanoculleus thermophilus* (relative abundance of 10% in T1 and T3, and 1% in T2) and *Methanothermobacter thermautotrophicus* (relative abundance varying between 2 to 26%) (Fig. 5b), respectively. Previous researches have reported the ability of the aforementioned species to efficiently produce methane under high TAN ($>7.0 \text{ NH}_4^+\text{-N L}^{-1}$) and FAN ($>0.9 \text{ g NH}_3\text{-N L}^{-1}$) level (Hao et al., 2015; Tian et al., 2018a). Furthermore, *M. thermautotrophicus* was also found with high abundance in reactors with ammonia-rich substrates, such as chicken manure (Niu et al., 2013).

The bacterial community of the thermophilic ammonia-tolerant consortia mainly consisted of three phyla: *Firmicutes*, *Synergistetes* and *Thermotogae* (Fig. 4c). Similar to the mesophilic consortia, 25% to 55% of the thermophilic bacteria were unable to be assigned at genus level (Fig. 4d). The most abundant OTU in all three consortia samples was *Anaerobaculum mobile* 5 (varying from 18% to 34%), which was reported to be a fermentative bacterium degrading peptides (Menes and Muxí, 2002) and can utilize acetate as carbon source to grow. Given that yeast extract (used in the medium) contains peptides and acetate was the main carbon source in this study, it could explain the thriving of the *A. mobile*. Additionally, several OTUs belonging to *Tepidmicrobium* spp., and several high abundant OTUs, such as MBA08 sp. 10 (90% similarity with *Hydrogenispora ethanolica*) and *Clostridiaceae* sp. 11 (90% similarity with *Caldicoprobacter* spp.) were also suspected to be related to the degradation of compounds contained in yeast extract (Niu et al., 2009; Bouanane-Darenfed et al., 2013; Liu et al., 2014).

Notably, the well-known SAOB *S. schinkii* was also detected with relative abundance varying between 1 to 6% in all the consortia. Moreover, the second most abundant OTU in T1, *Clostridia* sp. 6, had a 90% similarity to *Moorella humiferrea*, which was reported to be phylogenetically close to SAOB *T. phaeum* (Hattori et al., 2000). Similarly, the candidate genus S1 (including two OTUs and one was the second most abundant OTU in T3), which belonged to family *Thermotogaceae* and phylum *thermotogae*, was also suggested to perform SAO (Nobu et al., 2015). Taking all the above OTUs into account, the highest relative abundance of SAOB was found in T3, followed by T2 and T1, which is directly correlated with the relative abundance distribution of the hydrogenotrophic methanogens among the three consortia (T3>T1>T2).

4 Conclusions

This study revealed that aceticlastic methanogenesis was the dominant acetate methanogenic pathway at extremely high ammonia levels, mediated mainly by *M. soligelidi* and *M. thermophila* under mesophilic and thermophilic conditions, respectively. Surprisingly, strict methylotrophic *M. luminyensis* was the most abundant methanogen in mesophilic ammonia-tolerant consortia. This could be attributed to the unique *M. luminyensis* metabolism, which can use methylamine, produced from the amino acids, to form CH₄ and, at the same time, acetate only to produce biomass. However, even with dominant aceticlastic methanogenesis, SAOB and hydrogenotrophic methanogens (*Methanoculleus* spp. and *Methanothermobacter* spp.) were still detected, especially under thermophilic conditions.

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Appendix A. E-supplementary data

E-supplementary data of this article can be found in online version of the paper.

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Figure Legends

Fig. 1. Maximum specific growth rate ($\mu_{\max}$) of different inocula at different steps of the enrichment process. M1, M2, T1, T2 and T3 stands for Fangel, Hashøj, Blåhøj, Ribe and Snertinge. Error bars come from the triplicates of each setup.

Fig. 2. $^{14}\text{CO}_2/^{14}\text{CH}_4$ ratio of the ammonia-tolerant consortia based on radioactivity analysis. M1, M2, T1, T2 and T3 stands for Fangel, Hashøj, Blåhøj, Ribe and Snertinge. Error bars derived from the triplicate of each setup.

Fig. 3. Beta diversity of the triplicate samples of the ammonia-tolerant consortia based on principal component analysis. PCo1: principal coordinate 1. PCo2: principal coordinate 2. M1, M2, T1, T2 and T3 stands for Fangel, Hashøj, Blåhøj, Ribe and Snertinge, respectively.

Fig. 4. Taxonomic classification of the archaea at a) order and b) genus levels, and bacteria at c) phylum and d) genus levels of the ammonia-tolerant consortia. Phylum, and genus with relative abundance less than 0.5% were classified as “Others”. M1, M2, T1, T2 and T3 stands for Fangel, Hashøj, Blåhøj, Ribe and Snertinge.

Fig. 5. Relative abundance (with respect to the whole community) of interesting OTUs of a) mesophilic and b) thermophilic ammonia-tolerant consortia. M1, M2, T1, T2 and T3 stands for Fangel, Hashøj, Blåhøj, Ribe and Snertinge.

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Table 1. The characteristics of the used inocula and the operational parameters of the full-scale biogas plants, where the inocula derived from.

	Fangel	Hashøj	Blåhøj	Ribe	Snertinge
	(M1)	(M2)	(T1)	(T2)	(T3)
Temp. (°C)	37±1	37±1	53±1	53±1	53±1
HRT (days)	21	20	15	11	20
pH	8.10	7.92	7.88	7.93	7.77
VFA (mg L ⁻¹)	85.35±3.98	142.76±15.97	568.61±32.13	67.78±9.40	537.49±27.05
TAN (g NH ₄ ⁺ -N L ⁻¹)	4.68±0.04	3.99±0.07	4.12±0.14	3.11±0.12	3.67±0.05
FAN (g NH ₃ -N L ⁻¹)	0.65±0.01	0.39±0.01	0.86±0.02	0.71±0.03	0.62±0.01
TS (g L ⁻¹)	35.00±0.70	41.02±0.28	43.50±0.12	36.10±0.14	20.61±0.01
VS (g L ⁻¹)	21.70±0.23	24.63±0.22	29.18±0.11	23.72±0.15	13.57±0.06

* Temp: temperature; HRT: Hydraulic retention time; VFA: Volatile fatty acids; TAN:

Total ammonia; FAN: free ammonia; TS: Total solids; VS: Volatile solids.

Table 2. Experimental setup of the stepwise enrichment process

Enrichment	Mesophilic inocula			Thermophilic inocula		
Steps	TAN	pH	FAN	TAN	pH	FAN
	g NH ₄ ⁺ -N L ⁻¹		mg NH ₃ -N L ⁻¹	g NH ₄ ⁺ -N L ⁻¹		mg NH ₃ -N L ⁻¹
1	3.0	7.0	38.2	3.0	7.0	112.9
2	4.0	7.0	50.9	4.0	7.0	150.5
3	5.0	7.0	63.7	5.0	7.0	188.1
4	6.0	7.0	76.4	6.0	7.0	225.8
5	7.0	7.0	89.1	7.0	7.0	263.4
6	7.0	8.0	799.8	5.0	8.0	1405.5
7	9.0	8.0	1028.3	-	-	-

Highlights

- Acclimatization of methanogenic consortia to extreme ammonia levels
- *Methanomassiliicoccus luminyensis* consumes acetate to produce biomass but not CH₄
- Strict methylotrophic *M. luminyensis* was dominant at 37°C in acetate-fed reactors
- Aceticlastic pathway was dominant at extreme ammonia levels
- *Methanosarcina* spp. (*M. thermophila* & *M. soligelidi*) were vital at high FAN levels