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

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Abstract

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

Keywords

Biogas; Ammonia-tolerant consortium; Microbial community; *Methanoculleus thermophilus*; *Methanosarcina thermophila*.

1 Introduction

Many countries have recently introduced policies, aiming to increase the share of renewable energy in the total national energy consumption. Methane, a renewable energy carrier, produced through anaerobic digestion (AD) of organic materials, plays an important role in renewable energy supply (Kamali et al., 2016). Many nitrogen-rich biomasses such as chicken manure, aquaculture waste, slaughterhouse waste, microalgae, etc., are attractive AD feedstocks because of their high methane potentials (Tian et al., 2018b). The digestate from the AD of nitrogen-rich substrates is also classified as valuable biofertilizer due to its high levels of plant-available N nutrient (Möller and Müller, 2012). However, poor reactor performance with low methane production efficiency has been reported when nitrogen-rich substrates were used (Rajagopal et al., 2013). Within Europe Union (EU), there are more than 16,000 biogas plants with total installed electricity capacity higher than 10,100 MW (Scarlat et al., 2018). It was reported that some of these biogas plants lost more than 30% of their methane potential and consequently of their energy production, due to the high ammonia levels ($>3.0 \text{ g NH}_4^+ \text{-N L}^{-1}$) formed from the degradation of nitrogen-rich substrates (Angelidaki et al., 2005). In other words, the biogas industry is suffering significant economic losses due to ammonia problem. Therefore, it is urgent to develop a practical method to overcome ammonia inhibition and improve the energy production efficiency of the biogas reactors.

AD is mediated by different microorganisms, and is typically described as a four steps process, i.e. hydrolysis, acidogenesis, acetogenesis and methanogenesis (Angelidaki et al., 2011). The complex polymers are hydrolysed into different monomers during hydrolysis and consequently converted into different volatile fatty

acids (VFAs) during acidogenesis. Then acetate is mainly formed during acetogenesis from other VFAs, such as propionate (Eq. (1)) and butyrate (Eq. (2)). The electrons produced during the oxidation of propionate and butyrate are transferred to protons to generate hydrogen. It must be mentioned that these oxidation reactions are thermodynamically unfavourable and can only happen when the produced hydrogen is consumed by methanogens to keep a low level. Finally, methane is produced in methanogenesis from either oxidation of acetate (Eq. (5)) or reduction of CO₂ by H₂ (Eq. (4)), which is also identified as the most sensitive step to ammonia compared to other steps (Tian et al., 2018a). Acetate can be metabolized to methane in two ways: syntrophic acetate oxidation coupled with hydrogenotrophic methanogenesis (SAO-HM) (Eqs. (3) and (4)) and aceticlastic methanogenesis (AM) (Eq. (5)). Moreover, SAO-HM is believed to be more robust compared to AM at high ammonia levels (Sun et al., 2014; Werner et al., 2014; Tian et al., 2018a).

The total ammonia (TAN) exists as an equilibrium between ammonium ions (NH₄⁺) and free ammonia (NH₃, FAN) in the aqueous solutions. FAN, which increases along with pH and temperature, is the most toxic form of ammonia for the AD process (Massé

et al., 2014). Thus, at the same TAN levels, thermophilic (45-65°C) AD process usually faces much higher ammonia stress compared to mesophilic (30-40°C) process (Hansen et al., 1998). However, even with this drawback, thermophilic AD process still gains more and more attention in recent years, because of many advantages over mesophilic AD process. For example, thermophilic microorganisms usually have higher metabolic rates and higher growth rates compared to mesophilic ones, consequently result in shorter hydrolytic retention time (HRT) and higher organic matter removal rate (Weiland, 2010). Most importantly, thermophilic reactors are able to destruct the pathogenic microorganisms without the need of a pasteurization unit, which is necessary for the mesophilic reactors (Kim et al., 2002).

In recent years, bioaugmentation has been reported to alleviate ammonia inhibition in mesophilic continuously stirred tank reactor (CSTR) (Fotidis et al., 2014; Fotidis et al., 2017; Tian et al., 2019a). However, the basic bioaugmentation working mechanism and the microbiological interactions that allow a fast and successful bioaugmentation have not been clearly identified yet. Fotidis et al. (2014) proposed a “microbiological domino effect” theory, which the bioaugmentation culture triggered the growth of other ammonia-tolerant microorganisms. However, a solid microbial community dynamics during the ammonia alleviation process still needs to be investigated due to the lack of detailed bacterial information in the aforementioned study. The effect of the bioaugmentation culture on the indigenous microbial community of the reactors is still not clear so far, especially for the different bioaugmentation performance cause by the different bioaugmentation inocula. Additionally, bioaugmentation in thermophilic reactors is still a challenge compared to mesophilic reactors due to the higher ammonia stress, the higher sensitivity to the changes of the environment, and the lack of available

thermophilic ammonia-tolerant consortia, etc. (Yenigün and Demirel, 2013). Recently, a successful acclimatization to high ammonia levels (TAN=5.0 g NH₄⁺ L⁻¹, FAN=1.4 g NH₃ L⁻¹) of a thermophilic methanogenic consortium (mainly *Methanosarcina thermophila*, 95% relative abundance among the methanogens) and a pure hydrogenotrophic methanogenic strain (*Methanoculleus thermophilus*), was reported (Tian et al., 2018a; Tian et al., 2019b). This creates the opportunity to compare the different bioaugmentation inocula and decipher the interactions and/or the mechanisms that drive and define a successful bioaugmentation process.

Based on the above, the main goal of the current study was to identify the basic bioaugmentation working mechanism and analyse the microbial keystones of a fast and successful bioaugmentation. In order to realise the main goal, two preliminary goals were also considered: 1) to achieve, for the first time, successful bioaugmentation in thermophilic CSTR reactors and 2) to compare the performance and interactions of different bioaugmentation inocula. To realize these goals, two different ammonia-tolerant cultures (i.e. an enriched culture, and a mixed culture composed by *M. thermophilus* and the enriched culture) were used as bioaugmentation inocula to alleviate ammonia toxicity in different thermophilic CSTR reactors.

2 Material and methods

2.1 Inoculum and feedstock of the CSTR reactors

The inoculum used in this study to start-up the CSTR reactors was obtained from a lab-scale thermophilic (53±1 °C) CSTR reactor. Cattle manure, taken from Hashøj biogas plant (Denmark), was used as feedstock. The original manure was sieved with 1 mm diameter mesh, then stored in a freezer (-21 °C), and thawed in a thermostat (4 °C)

before use. The basic characteristics of the inoculum and the feedstock are shown in Table 1.

2.2 Bioaugmentation cultures

Two different bioaugmentation cultures were used in this study. The first culture was an enriched ammonia-tolerant consortium obtained from a previous study (Tian et al., 2019b). It was shown by the [2-¹⁴C] acetate radioisotopic analyses that aceticlastic methanogenic pathway was dominant in the consortium, which was mediated primarily by *Methanosarcina thermophila* (> 95% abundance among the methanogens). The cultivation of the enriched culture, prior to the bioaugmentation experiments, was conducted in batch bottles (238 and 40 mL total and working volume, respectively) with basal anaerobic medium (BA medium) and acetate (3.0 g HAc L⁻¹) as the cultivation medium and the main carbon source, respectively. The volatile suspended solid (VSS) concentration was 65 mg L⁻¹ at the end of cultivation. The detailed growth time course of the enriched culture is shown in the E-supplementary data. In order to achieve the same biomass concentrations (final VSS concentration of 332 mg L⁻¹) in all bioaugmentation tests, the enriched consortium was up-concentrated five times by centrifuging at 4500 rpm for 10 min under N₂ gas headspace.

The second bioaugmentation culture was a mixed culture consisting of a pure hydrogenotrophic strain (*Methanoculleus thermophilus* CR-1 (DSM No. 2373)) and the aforementioned enriched consortium (50/50, based on VSS). It must be mentioned that *M. thermophilus* and the enriched consortium were cultivated separately, and they were mixed together before the bioaugmentation process. The *M. thermophilus* CR-1 was purchased from DSMZ GmbH Company and cultivated as suggested by DSMZ (2017) with gas mixture of H₂ and CO₂ (80/20, v/v) as substrate. It was acclimatized to TAN

levels of 5.0 g $\text{NH}_4^+\text{-N L}^{-1}$ at pH 8.0 in batch bottles with 238 and 40 mL total and working volume, respectively. Moreover, the VSS concentration was determined to be 332 mg L^{-1} at the end of cultivation. The detailed growth of *M. thermophilus* is shown in the E-supplementary data.

2.3 Continuous reactor setup and bioaugmentation strategy

Three identical lab-scale CSTR reactors (R_{ctrl} , R_{enr} and R_{mix}) were used in the current study and each reactor had 2.3 and 1.8 L total and working volume, respectively. Each CSTR reactor consists of the reactor vessel with a magnetic stirrer, an influent bottle with a stirrer to ensure the substrate homogeneity, a peristaltic pump for feeding, an effluent bottle, an electrical heating jacket and a water-displacement gas meter. The reactors were operating under thermophilic ($53 \pm 1^\circ\text{C}$) conditions with HRT of 15 days, and organic loading rate (OLR) of 2.50 g VS $\text{L}^{-1} \text{d}^{-1}$ throughout the experiment.

The experimental period was divided into four different phases: P1 (days 0-13); P2 (days 14-20); P3 (days 21-23) and P4 (days 24-53). The reactors were running at a steady state (less than 10% methane production variation for at least ten consecutive days (Hansen et al., 1998)) at P1 with low ammonia levels (1.42 g $\text{NH}_4^+\text{-N L}^{-1}$) in the feedstock. At the beginning of P2, the TAN levels in the reactor were increased to 5.0 g $\text{NH}_4^+\text{-N L}^{-1}$ by spiking extra ammonia (ammonium chloride, NH_4Cl , CAS: 12125-02-9, Sigma-Aldrich) into both the reactor and the feedstock, and were kept constant (5.0 g $\text{NH}_4^+\text{-N L}^{-1}$) for all following phases (P2-P4). Bioaugmentation was performed in three consecutive days during P3, with 72 mL bioaugmentation culture each day in R_{enr} (only the enriched culture) and R_{mix} (the mixed culture). In total, each reactor was bioaugmented with 216 mL bioaugmentation inoculum, corresponding to 72 mg dry

biomass. By contrast, R_{ctrl} was used as control reactor (abiotic augmentation), where sterile BA medium and pure strain cultivation medium (50/50 v/v) were introduced.

2.4 Microbial analyses

To elucidate how the bioaugmentation culture affected the microbial composition both immediately and long term after bioaugmentation, four triplicate samples were taken from each reactor. The sampling times were at day 6 (before TAN increase, P1), day 17 (after TAN increase but before bioaugmentation, P2), day 26 (immediately after bioaugmentation, P4_a) and day 50 (two HRTs after bioaugmentation, P4_b). Genomic DNA was extracted with DNeasy PowerSoil® Kit (QIAGEN GmbH, Hilden, Germany). Moreover, the DNA concentration and quality were tested by NanoDrop 1000 (ThermoFisher). Polymerase chain reaction (PCR) amplification was performed on the V4 region of 16S rRNA gene with universal primers 515F/806R, and high throughput sequencing was performed by Illumina MiSeq platform (Ramaciotti Centre for Genomics, Kensington, Australia).

The raw sequencing reads, which were submitted to Sequence Read Archive (SRA) database (<http://www.ncbi.nlm.nih.gov/sra>) under the project No.SPR103296, were analysed by CLC Workbench (V.8.0.2) with microbial genomics module plug in (QIAGEN), as was described previously (Tian et al., 2018b). Sequencing depth was ensured by rarefaction curve (E-supplementary data). Furthermore, the taxonomical assignment of the interesting OTUs (relative abundance higher than 0.5% for bacteria and 0.01% for archaea, E-supplementary data) was validated through 16S ribosomal RNA sequences (Bacteria and Archaea) database on the website of National Center for Biotechnology Information. Furthermore, Multi Experiment Viewer software (MeV

4.9.0) (Saeed et al., 2006) was used to plot the relative abundance of the interesting OTUs as a heat map.

2.5 Physicochemical analyses

TS, VS, VSS, TKN and TAN were determined according to APHA (2012). The pH was measured by PHM99 LAB pH meter. Methane concentration and VFA levels in the reactors were measured through two different gas-chromatographs as were described before (Tian et al., 2018c).

2.6 Calculations and statistics

The OriginLab program (OriginLab Corporation, Northampton, Massachusetts) was used for statistical analyses and the preparation of the figures. The significantly changed ($p < 0.05$) OTUs between different samples were determined by STAMP software (Parks and Beiko, 2010).

3 Results and discussion

3.1 CSTR reactors' performance

3.1.1 Methane production efficiency

During P1, all the reactors performed similarly ($p > 0.05$) achieving a steady state with average methane yields of 163.6 ± 4.7 , 165.6 ± 3.8 and 167.5 ± 4.4 mL CH₄ g⁻¹ VS for R_{ctrl}, R_{enr} and R_{mix}, respectively (Fig. 1a). After TAN levels increased to 5.0 g NH₄⁺-N L⁻¹ (P2), the methane yield decreased rapidly and fluctuated with 34-39% methane production loss compared to the uninhibited P1. These methane production losses agreed with previous studies reporting that 30-39% methane reduction was observed in

223 thermophilic CSTR reactors when TAN levels increased up to $4.9 \text{ g NH}_4^+\text{-N L}^{-1}$ (Sung
224 and Liu, 2003; Tian et al., 2018c).

225
226 **Fig. 1.** The performance of the three reactors at different experimental phases: a)
227 methane yield, b) total VFA levels and c) pH. P1, P2, P3, P4 stands for the four
228 different experimental phases of the experiment, respectively.

Immediately after bioaugmentation (P4), the methane production of R_{mix} (bioaugmentation with the mixed culture) increased by 17% compared to P2 and established a steady state with an average yield of $130.3 \pm 4.2 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$ (78% recovery compared to P1) until the end of the experiment (Fig. 1a). R_{enr} , which was bioaugmented with only the enriched consortium, also increased its methane production after bioaugmentation, but it recovered the methane much slower than R_{mix} . Specifically, the methane production of R_{enr} started increasing three days after bioaugmentation, and it became similar to R_{mix} after another seven days, with an average methane yield of $133.0 \pm 3.7 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$ (80% recovery compared to P1). These results were in accordance with previous studies reporting a sharp methane production increase after bioaugmentation compared to the inhibited period (Fotidis et al., 2014; Fotidis et al., 2017). R_{ctrl} also increased its methane yield from $101.3 \pm 3.6 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$ during P2 to $117.3 \pm 3.8 \text{ mL CH}_4 \text{ g}^{-1} \text{ VS}$ at the last HRT of the experiment, indicating a certain degree of adaptation of the AD microbiome to the ammonia toxicity, but it was still significantly lower ($p < 0.05$) compared to R_{mix} and R_{enr} . This natural, slow adaptation of methanogenic cultures to high ammonia levels in continuous reactors have also been reported by many researchers (Calli et al., 2005a; Tian et al., 2018b).

In the current experiment, R_{enr} and R_{mix} improved the methane production efficiency by 11-13% compared to R_{ctrl} during the steady state period after bioaugmentation. Thus, for the first time bioaugmentation was applied successfully to alleviate ammonia inhibition in thermophilic CSTR reactors. The similar final improvement of methane production for both bioaugmented reactors (R_{mix} and R_{enr}) indicated the comparable levels of the available free energy in the two reactors that the bioaugmented microbiome could utilize at the extreme conditions. It has been reported by Rajagopal et al. (2013)

that high ammonia/ammonium levels could inhibit the methane producing enzyme thereafter increase the energy barrier of the methanogenic reactions. Thus, it seems that the available free energy levels were dictated by the effect of ammonia on the metabolic efficiency of the pre-existing microbiome members, and once the energy barrier was overcome by the bioaugmentation inocula, similar energy was released and recovered. Nonetheless, further investigation using pure carbon sources to pinpoint the free energy availability and the different metabolic bottlenecks are necessary. Another interesting observation was that the methane production was recovered much faster in R_{mix} than R_{enr} , manifested by 15% higher overall methane production during the first ten days after bioaugmentation. This indicates that the mixed culture was a better bioaugmentation inoculum compared to the enriched culture, which demonstrated the importance of hydrogenotrophic methanogens included in the mixed culture. A possible explanation for this behaviour is that the instant hydrogen partial pressure removal by hydrogenotrophic *M. thermophilus* created better thermodynamic conditions for the overall AD process (Hao et al., 2017).

3.1.2 VFA accumulation and pH fluctuation

In general, the VFA accumulation of each reactor matched with the methane production variations. Specifically, before increasing the ammonia levels, the VFA levels of all the reactors were inside the normal threshold ($<1500 \text{ mg L}^{-1}$) (Angelidaki et al., 2005) for CSTR reactors (Fig. 1b). As expected, the VFA levels increased rapidly after increasing ammonia levels and exceeded the 1500 mg L^{-1} threshold (Angelidaki et al., 2005) at the end of P3 for all the reactors. This VFA increase was reported before when CSTR reactors were exposed to increased ammonia levels (Westerholm et al.,

2012; Tian et al., 2017). Moreover, The VFA increase also supports that
methanogenesis was more susceptible to ammonia inhibition compared to acetogenesis.

The VFA levels in the R_{ctrl} kept increasing at the beginning of P4 and then
stabilized between 2200 to 2700 mg L⁻¹ throughout the rest of the experiment. In
contrast, the VFA increasing trend in R_{mix} ceased immediately after bioaugmentation
and stabilised around 1000 mg L⁻¹ until the end of the experiment, demonstrating the
immediate positive bioaugmentation effect of the mixed culture. While the VFA levels
in R_{enr} started decreasing three days after bioaugmentation from 2300 mg L⁻¹ to 1250
mg L⁻¹ at the end of P4, indicating a slower bioaugmentation effect on VFA reduction.
This faster VFA consumption that was observed in R_{mix} compared to R_{enr} , agreed with a
previous observation where acetate was consumed faster through SAO-HM (e.g. R_{mix} in
this study) compared to AM pathway (e.g. R_{enr} in this study) under stressful conditions
(Hao et al., 2011). Moreover, the changes in the accumulations of acetate and
propionate (Fig. 2) also supported the faster bioaugmentation effect scenario in R_{mix}
than in R_{enr} . Specifically, immediately after bioaugmentation, the acetate levels in R_{mix}
start decreasing followed by the propionate levels. In R_{enr} , the acetate levels started
dropping three days after bioaugmentation and reached similar levels compared to R_{mix} ,
while the propionate remained comparatively high but statistically ($p < 0.05$) lower than
 R_{ctrl} . Even though the dissolved hydrogen concentration was not directly measured in
the current study, it is generally accepted that increasing hydrogen partial pressure due
to the methanogenesis inhibition is the main reason for VFA accumulation (especially
propionate) during methanogenesis inhibition (Cord-Ruwisch et al., 1997; Björnsson et
al., 2001; Boe et al., 2010). Therefore, the VFA levels decreased in both reactors (R_{mix}
and R_{enr}) after bioaugmentation since aceticlastic *M. thermophila* was present. However,

due to the additional presence of hydrogenotrophic *M. thermophilus* in R_{mix} , the reactor had a faster hydrogen partial pressure reduction compared to R_{enr} , which resulted in an even faster VFA reduction. Additionally, this direct hydrogen consumption cut off the back step reaction (homoacetogenesis- accumulation of acetate) and made syntrophic acetate oxidation and other VFAs degradation processes (Eq. 1-5) thermodynamically favourable (Massé and Droste, 2000). To sum up, thermodynamic conditions of the overall AD process were improved through bioaugmentation, consequently increased the methane production and decreased the VFA accumulation. Overall, at the end of the experiment, the bioaugmented reactors, i.e. R_{enr} and R_{mix} , had 45-52% lower VFA levels compared to R_{ctrl} reactor.

Fig. 2. The changes of the individual VFA at different experimental phases: a) acetate levels and b) propionate levels. P1, P2, P3, P4 stands for the four different experimental phases of the experiment, respectively.

The pH levels fluctuated between 7.85 and 8.05 before the addition of ammonia (P1, Fig. 1c), and decreased to lower levels varying between 7.55 and 7.75 due to the VFA accumulation after ammonia addition (P2-P4) (Angelidaki and Ahring, 1993). However, thanks to the high buffer capacity of manure, even with fluctuations, the pH levels remained within the optimal pH range (6.5-8.5) for AD process (Nissilä et al., 2012).

3.2 Microbial community composition and dynamics

3.2.1 Microbiome overview

The alpha diversity (based on Chao 1 bias-corrected index (Zhu et al., 2017)) in R_{mix} decreased throughout the experiment to form a more specialized community (Fig. 3), which resulted in a more efficient AD process. However, the alpha diversity in R_{ctrl} and R_{enr} increased at P2 and P4a compared to P1; it seems that ammonia addition stimulated the growth of microbes that were less abundant during P1 and resulted in a temporarily high diversity (Werner et al., 2014). For both reactors, the diversity decreased at the end of P4, where species were eliminated from the reactors (washout effect (Tian et al., 2018b)) due to ammonia toxicity stress and more specialised communities were formed.

Fig. 3. Alpha diversity based on Chao 1 bias-corrected index at different phases of the experiment.

Fig. 4. Principal component analysis of the triplicate samples from different phases and reactors. PC1 and PC2 stand for principal component 1 and 2, which explain 66.3% and 17.0% of the community variation, respectively. P1, P2, P4_a and P4_b stands for the four sampling points, respectively: P1 at day 6 (before TAN increase), P2 at day 17 (after TAN increase but before bioaugmentation), P4_a at day 26 (immediately after bioaugmentation) and P4_b at day 50 (two HRTs after bioaugmentation).

Beta diversity based on principal component analysis (Fig. 4) showed clear microbial community dynamicity between the different experimental phases. At P1, all the samples clustered together indicating a high similarity of the microbial community of all the reactors. However, after the addition of ammonia, the microbial composition in all reactors started differentiating compared to P1. Specifically, an interesting finding was that immediately after bioaugmentation (P4_a), the triplicate samples from R_{mix}

clustered together and were significantly different from R_{ctrl} ($p < 0.05$) and R_{enr} ($p < 0.05$). This difference might contribute to the better performance of R_{mix} compared to R_{ctrl} and R_{enr} during that period. Overall, it was clear also by the current experiment that ammonia plays a decisive role in shaping anaerobic microbial communities (De Vrieze et al., 2015; Tian et al., 2018b); and that a new, ammonia-tolerant microbiome could be established rapidly through bioaugmentation (Fotidis et al., 2017) based on the improved reactor performance.

3.2.2 Communities dynamics

In general, the microbial community consisted of mainly bacteria, which accounted for more than 97% of the total relative abundance, and the rest were archaea. *Firmicutes* was the predominant phylum in all the samples (the relative abundance varied from 73% to 78%), followed by *Bacteroidetes* (13-15%), and other less abundant phyla, such as *Proteobacteria*, *Synergistetes*, *Thermotogae*, *Euryarchaeota*, etc. (E-supplementary data).

Regarding the changes of each individual OTU (Fig. 5), the first noteworthy OTU was *M. thermophilus* 2, which represented the hydrogenotrophic *Methanoculleus thermophilus* that was included in the mixed bioaugmentation culture. Its relative abundance decreased significantly ($p < 0.05$) in all reactors after ammonia addition (Fig. 5a-c). The decreased low abundance remained similarly during the rest periods in R_{ctrl} until the end of the experiment and increased slightly in R_{enr} to 2.9% (with respect to the archaea community) at P_{4b} , while it increased more than twofold in R_{mix} and reached 11.5% at P_{4b} after bioaugmentation. At the same time, an interesting observation was that two OTUs, *Thermoanaerobacteraceae* sp.17 and *Thermoanaerobacteraceae* sp.18, which were identified to be *Thermacetogenium phaeum* (93% similarity), decreased

significantly ($p < 0.05$) in all the three reactors after the addition of ammonia. However, it increased significantly in R_{mix} by 32% immediately after bioaugmentation, and reached a significant higher relative abundance (6.5%) compared to R_{ctrl} and R_{enr} . *T. phaeum* is a well-known syntrophic acetate oxidising bacterium (SAOB) (Hattori et al., 2000), which couples with hydrogenotrophic methanogens to mediate the SAO-HM pathway. Therefore, the current dynamics of the three aforementioned OTUs indicated that bioaugmentation with hydrogenotrophic *M. thermophilus* in R_{mix} stimulated the growth of *T. phaeum* thus resulted in faster syntrophic acetate oxidation (SAO) process and consequently recovered the methane production faster than the other two reactors. The result also highlighted the importance of hydrogenotrophic methanogens in the bioaugmentation process.

On the other hand, the relative abundance of *Methanosarcina thermophila* (dominant methanogen in the bioaugmented enriched culture) was very low in all reactors before bioaugmentation. After bioaugmentation, it increased significantly ($p < 0.05$) by more than twofold in R_{enr} and R_{mix} , but not in R_{ctrl} , verifying the successful establishment of the selected cultures in the continuous reactors through bioaugmentation. However, the high abundance of *M. thermophila* was detected only at $P4_a$ (i.e. short period immediately after bioaugmentation), and it almost disappeared at the end of the experiment. *Methanosarcina* spp. play an important role in consuming acetate when acetate accumulates in thermophilic reactors (Hori et al., 2006). Therefore, in the current study, it seems that *M. thermophila* thrived during the acetate accumulation period, and it was washed out due to interspecific competition (Tian et al., 2018b) when the acetate levels went below 1000 mg HAc L⁻¹ (E-supplementary data).

As a result, the enriched consortium triggered the establishment of the new and specialised community with efficient biogas productivity at high ammonia levels.

Throughout the experimental period, the most dominant methanogenic OTU was always *Methanothermobacter* sp.1 in all three reactors. The relative abundance of *Methanothermobacter* sp.1 varied between 60 to 83% (total methanogens) at different phases (Fig. 5a-c), verifying a well-documented strong hydrogenotrophic methanogenic activity of *Methanothermobacter* spp. in thermophilic reactors (Yabu et al., 2011; Franke-Whittle et al., 2014; Kougias et al., 2017). Moreover, the relative abundance of *Methanothermobacter* sp.1 decreased significantly ($p<0.05$) after the addition of ammonia, but it increased back during P4 up to the initial levels (P1) for reactors R_{ctrl} and R_{enr}, indicating its ability to adapt at high ammonia levels (Niu et al., 2013). However, although *Methanothermobacter* sp. kept being dominant, its relative abundance in R_{mix} decreased significantly ($p<0.05$) at P4_b, which could be explained by the increased abundance of bioaugmented *Methanoculleus thermophilus*, as discussed above.

Fig. 5. Relative abundance shown as heat map for the interesting archaea and bacteria at different phases in different reactors: a) archaea in R_{ctrl} , b) archaea in R_{enr} , c) archaea in R_{mix} , d) bacteria in R_{ctrl} , e) bacteria in R_{enr} and f) bacteria in R_{mix} .

Another interesting finding was that OTU *Methanosarcina* sp.5 increased its relative abundance by more than twofold after ammonia addition in all the reactors. This could be due to the ability of *Methanosarcina* spp. to form aggregates, which might create an ammonia gradient to the core of the aggregate, thus reduce the ammonia

toxicity to some extent (Calli et al., 2005b; Goberna et al., 2010). However, the rapid decreased relative abundance of *Methanosarcina* sp.5 during the following phases demonstrated its limited long-term tolerance to high ammonia toxicity.

Finally, the bioaugmentation also triggered changes of the most abundant bacteria group, i.e. the uncultured MBA08 that included *Clostridiaceae* sp.13, *Clostridiaceae* sp.15, *Clostridiales* sp.21 and MBA08 sp.50. The relative abundance of MBA08 increased slightly from 30% to 31% in R_{ctrl} , but significantly from 31% to 34% in R_{enr} and from 28% to 35% in R_{mix} , before and after bioaugmentation, respectively. MBA08 was previously reported to be dominant in thermophilic reactors operated at high ammonia levels (De Vrieze et al., 2015) and was suspected to involve in cellulose degradation (Sun et al., 2015). It seems that the high fibre content in the feedstock (cattle manure) contribute to the high abundance of MBA08 in this study, and the significant increase of its abundance in R_{enr} and R_{mix} was in accordance with the high methane production after bioaugmentation. However, the aforementioned discussion was derived by a DNA-based methodology (i.e. 16r rRNA gene sequencing) and as De Vrieze et al. (2016) has pointed out, the microbial abundance does not necessarily reflects the microbial activity. Thus more advanced microbiology, such as RNA-based metatranscriptomics, is suggested for future studies correlating microbial community and activity.

3.3 A proposed mechanism of the bioaugmentation effect

Overall, the immediate reduction of the hydrogen partial pressure by the bioaugmented hydrogenotrophic methanogen seemed to be the main reason for a fast and more efficient bioaugmentation. Even though both bioaugmentation inocula resulted in an improved reactor performance, the mixed culture had a faster effect in

recovering the methane production compared to the enriched culture, which indicated that the hydrogenotrophic *M. thermophilus* was the key to a successful bioaugmentation effect.

Fig. 6. Proposed mechanisms depicting the effects of bioaugmentation on the three reactors. The blue solid and dashed arrows present the conversion direction and decrease of the compounds' concentration, respectively, and two arrows stand for stronger effect compared to the single arrow. The orange and green processes stand for the reactors' performance before and after bioaugmentation, respectively.

Based on the evaluation of all operational and microbiological data, a potential bioaugmentation working mechanism was proposed (Fig. 6). Specifically, the instantly decreased hydrogen partial pressure, due to the consumption by *M. thermophilus* in

R_{mix} , ceased homoacetogenesis, increased SAO and created favourable conditions (Eq. (1), (2), (4)) for VFA degradation (Massé and Droste, 2000; Hao et al., 2017) by enhancing acetogenesis (especially from propionate, E-supplementary data). Meanwhile, the enriched consortium (mainly aceticlastic *M. thermophila*) in the mixed culture also degraded acetate directly. Therefore, the VFA-ammonia synergistic inhibitory effect (Lu et al., 2013) was alleviated rapidly due to the reduction of the hydrogen partial pressure, which resulted in a faster methane recovery compared to the other reactors. When only the enriched culture was used (R_{enr}), it seems that it mainly decreased acetate degradation (Fig. 2a) through AM pathway, but it did not significantly contributed to the hydrogen partial pressure reduction. This resulted in a slower acetogenic step from other VFAs. For example, propionate levels did not decrease significantly in R_{enr} after bioaugmentation (Fig. 2b). Therefore, both strategies alleviated ammonia inhibition, but the bioaugmentation only with the enriched culture did not recover the methane production as fast as the bioaugmentation with the mixed culture.

4 Conclusions

The results demonstrated the basic mechanism that drives a successful and fast bioaugmentation process in ammonia inhibited CSTR reactors. This mechanism includes/requires the instant hydrogen partial pressure reduction by bioaugmented ammonia-tolerant hydrogenotrophic methanogens that allows a thermodynamic SAO process, which consequently decreases the VFA levels (reduction of the VFA-ammonia synergistic inhibitory effect) and thus, improves the overall AD process. Furthermore, it showed for the first time that bioaugmentation with ammonia-tolerant cultures can alleviate ammonia inhibition in thermophilic continuous reactors. Overall, the

comparison between the different bioaugmentation inocula highlighted the crucial role of hydrogenotrophic methanogen in this process.

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Appendix A. Supplementary material

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

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