Inhibitory effects of ammonia on methanogen mcrA transcripts in anaerobic digester sludge

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Abstract
Methanogens in anaerobic ammonia-rich digesters show differential responses to ammonia stress. The mechanism for this is poorly understood. In the present study, we determined the rates of methane production, the composition of methanogen mcrA (the gene coding for the alpha subunit of methyl-coenzyme M reductase) and their transcripts in response to ammonium addition in the anaerobic sludge retrieved from a full-scale digester treating swine manure. The rate of CH4 production substantially reduced with increased addition of ammonium. The analysis of natural 13C abundances of CH4 and CO2 indicated that the aceticlastic methanogenesis was more sensitive than hydrogenotrophic methanogenesis. Quantitative PCR analysis revealed that mcrA copy number decreased by one order of magnitude in the treatment with a large amount of ammonium (10 g NH4+-N L−1) but did not change much with treatments of smaller amounts (3 and 7 g NH4+-N L−1) compared with the control. T-RFLP analysis of mcrA compositions showed that the structure of the methanogen community remained highly stable, with Methanosaetaceae dominating the methanogen community in all incubations. The composition of mcrA transcripts, however, showed a substantial response to the addition of ammonium. The relative abundance of Methanosaetaceae transcripts declined with increasing amounts of ammonium, whereas the transcript level of Methanobacteriales mcrA was relatively resistant. The differential responses corresponded to the shift of methanogenic pathway inferred from 13C isotope fractionation. Our study suggests that methanogens in anaerobic sludge have a strong mcrA transcriptional response to ammonia stress without a change in the community structure.

Introduction
Anaerobic digestion is a useful technology for energy recovery, which helps reduce the dependence on fossil fuels, mitigate the emission of greenhouse gases, and lower the risks of environmental pollution (McKendry, 2002; Amani et al., 2010). In addition, the digestion residues, normally containing various nutrients, can be used as agricultural fertilizers. Different factors influence the performance of anaerobic digesters and a few of them can cause the reactor upset or failure (Chen et al., 2008; Amani et al., 2010). Accumulation of ammonia, produced from the degradation of protein-rich materials, is one such detrimental factor in the anaerobic digesters used to treat waste such as livestock and poultry manure (Van Velsen, 1979; Zeeman et al., 1985; Wiegent & Zeeman, 1986; Chen et al., 2008; Garcia & Angenent, 2009).

The free ammonia that can diffuse readily across cell membrane is considered the main form of ammonia/ammonium toxicity (Sprott & Patel, 1986; Kadam & Boone, 1996). Environmental factors such as pH and temperature can affect the ammonia toxicity in anaerobic digesters by regulating the ammonia/ammonium equilibrium (Zeeman et al., 1985; Koster & Koomen, 1988; Kadam & Boone, 1996; Garcia & Angenent, 2009). Methanogens in anaerobic digesters exhibit different reactions to ammonia stress. In general, aceticlastic methanogens are more sensitive than the hydrogenotrophic methanogens.
Response of methanogen \textit{mcrA} transcripts to ammonia

(Sprott & Patel, 1986; Schnürer et al., 1994). For example, it has been demonstrated that the populations of acetotrophic methanogens declined whereas the hydrogenotrophic guilds increased when total ammonium concentration reached to > 3 g NH$_4^+$-N L$^{-1}$ in anaerobic digesters (Angenent et al., 2002; Westerholm et al., 2011). The accumulation of acetate and the suppression of acetotrophic methanogens with increasing ammonia favor the development of syntrophic acetate oxidation (SAO; Schnürer et al., 1999). A few syntrophic acetate oxidizers isolated from anaerobic digesters were indeed found tolerant up to 8 g NH$_4^+$-N L$^{-1}$ at neutral pH (Schnürer et al., 1996; Westerholm et al., 2011). Therefore, CH$_4$ production from acetate could shift from a pathway of mainly acetotrophic methanogenesis to SAO in combination with hydrogenotrophic methanogenesis (Schnürer et al., 1994; Westerholm et al., 2011, 2012). It was revealed, however, that the sensitivity of individual methanogens varied depending on digester conditions. For instance, Methanospirillum 	extit{sarcina} spp. were found to be either sensitive (Angenent et al., 2002; Westerholm et al., 2011) or tolerant to ammonia stress (Fotidis et al., 2013) in different digesters. Similarly, hydrogenotrophic methanogens showed changing responses in different experiments (Wiegant & Zee-Man, 1986; Westerholm et al., 2012; Fotidis et al., 2013). Apparently, the inhibitory effects of ammonia on individual methanogens remain poorly understood.

To investigate the inhibitory effects of ammonium on methanogens in anaerobic digester sludge, we collected a sludge sample from a full-scale swine manure digester and conducted a laboratory microcosm experiment. The responses of methanogenesis and methanogenic populations to ammonium addition were determined by analyzing the rate of CH$_4$ production, the $^{13}$C isotope fractionation of CH$_4$ and CO$_2$, and the copy number and transcripts of methyl-coenzyme M reductase encoding genes (\textit{mcrA}) in the sludge incubations. The methyl-coenzyme M reductase is the key enzyme metabolizing the last step of CH$_4$ production in all known methanogens and \textit{mcrA} has been used as a molecular marker for the phylogenetic analysis of methanogenic populations in environments.

Materials and methods

Anaerobic incubation

Activated sludge was collected from a full-scale anaerobic bioreactor (continuous stirred-tank reactor) treating swine manure in a scaled livestock farm located in Bei Langzhong Village, Shunyi District, Beijing. Anaerobic incubations were prepared by adding 10 g sludge and 1 g swine manure (wet weight) into 30 mL of 50 mM Hepes buffer (N-2-hydroxyethylpipерazine-N’-2-ethanesulfonic acid; Sprott & Patel, 1986) in 100-mL glass bottles. The total solid (TS) and volatile solid (VS) contents were 17.5% and 8.3% for sludge, and 29.7% and 25.0% for fresh pig manure, respectively. The buffer was added with NH$_4$Cl to create a final concentration of 0, 3, 7, 10 g NH$_4^+$-N L$^{-1}$ in incubation medium, respectively (hereafter referred to as 0-, 3-, 7- and 10-N treatments). No NH$_4$Cl was added to the control (0-N) but NH$_4^+$ was detected in the incubation due to organic matter mineralization. The buffer was autoclaved and pH was adjusted to 7.0 with 1 M HCl or NaOH prior to preparation. The bottles were sealed with butyl stoppers and flushed with N$_2$ for 3 min and then incubated at 35 °C in the dark. The experiments were carried out in triplicate.

Chemical analyses

The gaseous samples were taken from headspace with a pressure-lock precision analytical syringe (Baton Rouge, LA) and the concentrations of H$_2$, CH$_4$ and CO$_2$ were analyzed using GC-7890A (Agilent Technologies) equipped with TCD, FID and ECD. The $^{13}$C abundance (δ$^{13}$C) of CH$_4$ and CO$_2$ was analyzed by a gas chromatography-isotope ratio mass spectrometry system (Yuan & Lu, 2009). Liquid samples were collected and centrifuged as described previously (Rui et al., 2009). The supernatants were passed through 0.22-μm (pore size) filters (Sangon, Shanghai, China) and stored at −20 °C. Acetate was analyzed with an HPLC-1200 using a Zorbax SB-AQ C18 column (Agilent Technologies). The concentration of NH$_4^+$-N was analyzed colorimetrically using AUTOANALYZER 3 (Bran + Luebbe, Germany) and ammonia N (NH$_3$-N) was calculated based on NH$_4^+$-NH$_3$ equilibrium. The pH of incubation medium was measured directly using a pH meter (PB-10; Sartorius, Germany).

Nucleic acid extraction and cDNA synthesis

Sludge samples were taken from incubations at different time points. Microbial nucleic acids were extracted following the previous protocol (Noll et al., 2005; Ma et al., 2012) with modifications. Briefly, c. 2 g wet sludge was extracted first with 700 μL TPM buffer [50 mM Tris-HCl (pH 7.0), 1.7% polyvinylpyrrolidone K25, 20 mM MgCl$_2$, 1% sodium dodecyl sulfate] and then three times with 700 μL phenol-based lysis buffer [5 mM Tris-HCl (pH 7.0), 5 mM Na$_2$-EDTA, 1% sodium dodecyl sulfate, 6% water-saturated phenol]. Beads-beating was performed in FastPrep-24 (MP Biomedicals). The supernatants were further extracted with phenol, phenol-chloroform-isoamyl alcohol (25 : 24 : 1) and chloroform-isoamyl alcohol.
(24 : 1). The extracts were purified with cold ethanol and sodium acetate (Peng et al., 2008). RNA was obtained by removal of co-extracted DNA with RNase-Free Dnase Set (TaKaRa, China) at 37 °C for 30 min. RNA samples were purified using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and the removal of DNA was confirmed by the absence of PCR products after amplification of 16S rRNA genes with the universal primers for bacteria (27f/907r) and archaea (1096f/934r) (Peng et al., 2008; Rui et al., 2009). Reverse transcription PCR (RT-PCR) was performed using cDNA Synthesis Kit (TaKaRa), following the protocol described previously (Lueders & Friedrich, 2002; Yuan et al., 2011; Ma et al., 2012).

T-RFLP, cloning, sequencing and phylogenetic analyses

Both DNA and RNA (after cDNA synthesis) samples were used for PCR amplification of methanogen mcrA with the primer pair of MCRf and MCRr (Lueders et al., 2001). For terminal restriction fragment length polymorphism (T-RFLP) analysis, the forward primer was labeled with 6-carboxyfluorescein (FAM). The PCR products were purified and digested with BmgT 120 I (TaKaRa) at 37 °C for 3 h following the procedure described by Yuan et al. (2011) and Ma et al. (2012). The digested products were purified and the DNA fragments were analyzed using 3730xl DNA analyzer (Applied Biosystems) (Yuan & Lu, 2009). The relative abundance of terminal restriction fragments (T-RFs) was calculated as the percentage of each T-RF in total fluorescence intensity of a given T-RFLP profile. The minor T-RFs (relative abundance < 1%) were excluded in the analysis. Principle component analysis (PCA) of T-RFLP profiles was performed using CANOCO 4.5 software (Microcomputer Power, Ithaca, NY).

Three mcrA clone libraries were constructed with one from the DNA sample (day 6 in the 7-N treatment) and two from transcript samples (day 6 in the 3-N treatment and day 86 in the 7-N treatment). For cloning and sequencing, the same PCR procedure was performed without FAM labeling of primers. In total, 136 randomly selected clones were sequenced using pGEM-T Easy Vector system (Promega) according to the procedure described (Peng et al., 2008; Rui et al., 2009). The sequences were assigned to operational taxonomic units (OTUs) based on a sequence similarity cutoff of 99% using the DOTUR program (Schloss & Handelsman, 2005). Phylogenetic trees were constructed using neighbor-joining algorithm of the MEGA 4 program (Tamura et al., 2007). All sequences have been deposited into GenBank database under accession numbers JX860703–JX860838.

Quantitative analysis of mcrA copy number

The mcrA copy number was determined using quantitative (real-time) PCR in a 7500 real-time PCR system (Applied Biosystems) with the mlas/mcrA-rev primer pair (Steinberg & Regan, 2008), following the procedure described by Ma et al. (2012). The serial dilution and melting curve analyses were performed to optimize the qPCR parameters. The dilution rate of 100 : 1 was used to prevent the PCR inhibition for most samples. A standard curve was performed from the purified plasmid DNA carrying mcrA with the concentration ranging from 1.0 × 10⁵ to 1.0 × 10⁸ copies µL⁻¹. Each measurement was performed in triplicate. The raw data were logarithmically transformed and subjected to the parametric ANOVA analysis to test significant differences between treatments using the PSAW 18 software (SPSS Inc., Chicago, IL).

Results

Methanogenesis

The pH value of incubation medium decreased slightly from pH 7.0 to around pH 6.8 after 2 weeks of incubation (Fig. 1a). The pH stabilized except in the 10-N treatment, where it decreased further to about pH 6.7. The increase of acidity was in accordance with the decomposition of organic matter and the transient accumulation of fatty acids. The 10-N treatment showed a highest accumulation of acetate (Fig. 1d) and hence the lowest pH value. The Hepes buffer stabilized the pH values between pH 6.9 and 6.7 in most incubations. These pH values are within the physiological range of most methanogens in anaerobic digesters (Liu et al., 2008; Amani et al., 2010). The concentration of NH₃-N that varied according to experimental treatments did not change much over the experimental period (Fig. 1b). The free ammonia, however, decreased in the early stage in accordance with pH decrease (Supporting Information, Fig. S1). The quantity of free ammonia did not exceed 1.1% of total NH₃-N according to NH₃⁺/NH₃ equilibrium at 35 °C. On average, the concentrations of free ammonia were 3 mg N L⁻¹ for 0-N, 21 mg N L⁻¹ for 3-N, 49 mg N L⁻¹ for 7-N and 64 mg N L⁻¹ for 10-N, respectively. It has been shown that 10–30 mg NH₄⁺/N L⁻¹ is the critical range for ammonia effects on methanogens (Sawayama et al., 2004; Schnürer & Nordberg, 2008). Thus, the four treatments cover none to serious ammonia effects.

Hydrogen showed a transient accumulation at the beginning in the 7- and 10-N treatments. From day 7 until day 57, H₂ partial pressure was decreased to below the detection limit (about 6 Pa) in most incubations.
At the end of incubation, H₂ accumulated slightly in high NH₄⁺-N treatments. Acetate showed a rapid accumulation in the 0- and 3-N treatments but decreased to the detection limit (about 40 µM) from day 3 onwards (Fig. 1d). In 7-N treatment, acetate increased to 12 mM on day 6, leveled off between days 15 and 37, and then decreased to < 0.3 mM at the end of incubation. The concentration of acetate in the 10-N treatment kept increasing during the experiment, reaching 25 mM at the end of incubation (corresponding to 0.75 mmol CH₄ after complete conversion).

Methane production initiated without lag in the 0- and 3-N treatments showed a rapid phase in the beginning and then a slowdown phase after 20 days (Fig. 1e). The production rate in the rapid phase was 27% lower in the 3-N treatment than in the 0-N control. Methane production was significantly inhibited in the 7-N treatment, but a slow rate was detected throughout the experiment. Methane production in the 10-N treatment was strongly repressed over the experimental period. Only in the last days of experiment was a low rate of production detected. The concentration of acetate in the 10-N treatment kept increasing during the experiment, reaching 25 mM at the end of incubation (corresponding to 0.75 mmol CH₄ after complete conversion).

Methanogen dynamics

To determine the composition of the methanogen community in sludge incubations, we constructed three mcrA clone libraries. Phylogenetic analysis of 136 clone sequences indicated that the methanogen community comprised mainly Methanosaetaceae, Methanosarcinaceae, Methanospirillaceae, Methanomicrobiaceae and Methanobacteriaceae (Fig. 2). Methanosaetaceae predominated in most samples, whereas Methanomicrobiaceae showed only a minor abundance (Table 1). Clone sequences were used to relate T-RFs in the T-RFLP fingerprints to the respective methanogens. The following assignments were obtained: 254 bp with Methanosacetaeae; 393 and 493 bp with Methanosarcinaceae; 496 bp with Methanospirillaceae; 408 and 469 bp with Methanobacteriaceae (Fig. 2, Table 1). The 429-bp T-RF was indicative of both Methanospirillaceae and Methanomicrobiaceae.
T-RFLP analysis of both mcrA compositions and their transcripts was conducted at different time points (Fig. 3). Measurements of CH\(_4\) and substrates H\(_2\) and acetate indicated that methanogenesis in the 0- and 3-N treatments reached a quasi-steady state 3 weeks after incubation (Fig. 1) and therefore T-RFLP analysis was not performed further for these treatments after day 31. T-RFLP of mcrA composition revealed that the

### Table 1. Phylogenetic affiliations and numbers of methanogen mcrA sequences retrieved in clone libraries

<table>
<thead>
<tr>
<th>Phylogenetic group</th>
<th>mRNA (3C6d* )</th>
<th>mRNA (7A86d* )</th>
<th>DNA (7A6d* )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>T-RF (bp)</td>
</tr>
<tr>
<td>Methanobacteriaceae</td>
<td>9</td>
<td>17</td>
<td>408, 469</td>
</tr>
<tr>
<td>Methanomicrobiaceae</td>
<td>1</td>
<td>2</td>
<td>429</td>
</tr>
<tr>
<td>Methanospirillaceae</td>
<td>15</td>
<td>29</td>
<td>429, 496</td>
</tr>
<tr>
<td>Methanosarcinaceae</td>
<td>16</td>
<td>36</td>
<td>393</td>
</tr>
<tr>
<td>Methanosaetaeaeae</td>
<td>27</td>
<td>52</td>
<td>254</td>
</tr>
<tr>
<td>Total clones</td>
<td>52</td>
<td></td>
<td>408, 469</td>
</tr>
</tbody>
</table>

*The mcrA clone libraries were obtained from one DNA sample collected on day 6 in the sludge treated with 7 g NH\(_4\)\(_2\)N g\(^{-1}\) ammonium (referred to as 7A6d) and two mRNA samples collected on day 6 in the sludge treated with 3 g NH\(_4\)\(_2\)N g\(^{-1}\) ammonium (referred to as 3C6d), and on day 86 in the sludge treated with 7 g NH\(_4\)\(_2\)N g\(^{-1}\) ammonium (referred to as 7A86d), respectively.†Relative abundance (%).
community structure of methanogenic archaea in the sludge incubations remained highly stable across incubations (Fig. 3a–d). The 254-bp T-RF, indicative of Methanosaetaceae, predominated in all samples, accounting for >70% of the total fluorescence intensity in T-RFLP profiles. The next major T-RF (496-bp), indicative of Methanospirillaceae, accounted for 5–20% of total fluorescence intensity in T-RFLP profiles. The 408- or 469-bp T-RF, indicative of Methanobacteriaceae, showed only a minor abundance (<10%) in the DNA-based analysis. T-RFLP patterns were generally not affected by the addition of ammonium in comparison with the control. But it appears that the composition of the methanogen community became more diverse with the increase in ammonium (Fig. 3c and d).

T-RFLP patterns of mcrA transcripts differed markedly from the gene compositions. The relative abundances of 408- and 469-bp T-RFs substantially increased, whereas that of 254-bp T-RF decreased in mcrA transcript profiles. In the 0-N control, the increase of 408-bp T-RF was detected only in the early stage, and the proportion of 254-bp T-RF increased gradually with time (Fig. 3e).
Addition of ammonium magnified the difference between the transcripts and gene compositions. In the 3-N treatment, the 408- and 496-bp T-RF increased more than the control (Fig. 3f). But the 254-bp T-RF still showed a tendency to increase with time. In the 7- and 10-N treatments, the 408-bp T-RF remained predominant throughout the experiment, accounting for nearly a half of the total fluorescence in all samples (Fig. 3g and h). In addition, the 393-bp T-RF, indicative of Methanosarcinaceae, markedly increased towards the end of incubation, but the relative abundance of 254-bp T-RF continued to decrease during the experiment.

We performed PCA analysis for all T-RFLP data from DNA and mRNA samples (Fig. 4). DNA samples were clustered closely, indicating the close similarity of methanogen compositions among different samples. The mRNA samples, however, differed from the DNA samples, which were scattered due to ammonium treatments. Thus, DNA samples did not reflect the difference in treatment, whereas the mRNA samples revealed the clear effects of ammonium addition.

The total mcrA copy numbers as estimated by quantitative PCR ranged from $4.5 \times 10^9$ to $6.6 \times 10^{10}$ copies g$^{-1}$ sludge (dw) in anaerobic incubations (Fig. 5). The copy numbers decreased with time in the early stage in the 0-, 3- and 7-N treatments, but there was no significant difference among these treatments (Fig. S2). In the 10-N treatment, the mcrA copy numbers decreased from $6.1 \times 10^{10}$ copies g$^{-1}$ sludge (dw) at the beginning to $4.5 \times 10^9$ copies g$^{-1}$ sludge (dw) on day 22. The copy numbers then remained low, albeit with a slight increase at the end. On average, the mcrA copy numbers in the 10-N treatment was decreased one order of magnitude compared with the 0-N control.

**Discussion**

Our study demonstrated that the addition of ammonium resulted in inhibition of CH$_4$ production in anaerobic sludge, in agreement with previous studies (Van Velsen, 1979; Wiegant & Zeeman, 1986; Schnürer et al., 1999, Sawayama et al., 2004; Westerholm et al., 2012; Fotidis et al., 2013). We determined the carbon isotope abundance of CH$_4$ and CO$_2$. The carbon isotope of acetate was not measured, but we assumed that its isotope ratio did not vary among incubations because the same sludge and manure materials were used. The $\delta^{13}$C values of CO$_2$ remained constant in incubations. Thus, the change in the $\delta^{13}$C value of CH$_4$ presumably reflected the isotopic discrimination of hydrogenotrophic vs. aceticlastic methanogenesis (Conrad, 2005). The low $\delta^{13}$C value in the treatment with a large amount of ammonium (10-N) indicated that CH$_4$ production, if occurring, was primarily via the hydrogenotrophic pathway. The fluctuation of the $\delta^{13}$C value in the 7-N treatment suggested a suppression of aceticlastic methanogenesis in the early stage followed by its resumption in the later period. This was corroborated by the change of acetate concentration in the sludge slurries (Fig. 1d). The high and stable $\delta^{13}$C values in the control and low ammonium treatment (3-N) indicated that CH$_4$ was mainly produced by aceticlastic methanogenesis, coinciding with the prevalence of Methanosetaeae and the low concentration of acetate in these incubations. We should note, however, that the pattern of carbon isotope disproportion may differ from the
theoretical prediction, as the fermentation pathway leading to acetate metabolism and methane production could change with ammonium treatment. For instance, the accumulation and syntrophic oxidation of propionate may suppress SAO, allowing acetate to remain in the medium. Alternatively, the upstream fermenting microbes might respond differently and hence the carbon/electron flow through the food web was altered after ammonium treatment. All changes in the upstream pathways can influence the final pattern of carbon isotope disproportion. Nevertheless, our results revealed that aceticlastic methanogenesis was markedly inhibited, whereas hydrogenotrophic methanogenesis was relatively resistant to ammonium stress. The adaptation and recovery of aceticlastic methanogenesis can occur if ammonium concentrations do not exceed 7 g NH$_4^+$-N L$^{-1}$.

We analyzed the response of the methanogenic community based on mcrA T-RFLP fingerprints. It has been shown that mcrA primers may under-represent methanogens within the order of Methanomicrobiales due to primer degeneration (Lueders & Friedrich, 2003). Despite this bias, the systematic comparison of DNA and RNA fingerprints is still valid for distinguishing the treatment effect. Our results showed that the methanogen community structure as determined by DNA-based T-RFLP remained stable. *Methanoseta spp.* dominated the methanogen community in the sludge and this pattern did not change with the addition of ammonium (Fig. 3a–d). Addition of ammonium did not affect the total methanogen population except with for the treatment with the highest amount of ammonium (10-N) where the population size was markedly decreased compared with the lower ammonium treatments.

Methanogenic populations in the system were derived mainly from the sludge slurry and probably partly from the fresh pig manure. The stability of the methanogen community was probably due to the limited growth during the incubation. Alternatively, the batch incubation in the present experiment would not allow the washout of inactive organisms, and hence both active and inactive populations were counted in the analyses. It has been considered that the community represented by DNA fingerprints includes a pool of existing methanogens, but only a small fraction of the pool might be active and react to environment changes (Yuan et al., 2011).

The T-RFLP analyses of mRNA showed distinct patterns from DNA fingerprints (Fig. 4). The results revealed a substantial response of mcrA transcripts to ammonium addition. The relative abundance of *Methanosetaeaceae* transcripts (254 bp) markedly declined, whereas that of *Methanobacteriales* (408 bp) increased after ammonium addition. The increase of *Methanobacteriales* transcripts is remarkable given that these methanogens showed only a minor abundance in the gene profiles (Fig. 3). The repression of *Methanosetaeaceae* depended on ammonium concentration, being recoverable at 3 g NH$_4^+$-N L$^{-1}$ (Fig. 3f). While *Methanosetaeaceae* were constantly repressed in the higher ammonium treatments, *Methanosarcinaceae* (393 bp), with a very low relative abundance in the gene profiles, showed a marked increase in the transcript profiles towards the end of incubation (Fig. 3g and h). We would like to indicate here that the precise comparison of community dynamics might be not valid due to the halt of analyses in the control and 3-N treatment after day 31. The assumption that the community has reached to a steady state in these treatments requires further validation.

The response of methanogen mcrA transcripts was closely related to the methanogenic activities. Aceticlastic methanogenesis dominated CH$_4$ production in the incubations without ammonium addition. The repression of *Methanosetaeaceae* transcripts in the high ammonium treatments thus resulted in significant accumulation of acetate. The relative resistance of *Methanobacteriales* transcripts coincided with the domination of the hydrogenotrophic pathway, although at a very low rate of total CH$_4$ production. *Methanosarcinaceae* were activated in the later stage and were probably responsible for the resumption of aceticlastic methanogenesis. Alternatively, SAO could be suppressed due to the accumulation of fermentation products other than acetate. The partial pressure of H$_2$, occurring at the end of incubations, might be relatively elevated and thus could suspend SAO during the syntrophic oxidation of fermentation products such as propionate and butyrate.

The pattern of mcrA transcript responses to ammonium addition is in line with previous observations on methanogenic activities in pure cultures (Sprott & Patel, 1986; Steinhaus et al., 2007) and *in situ* anaerobic digesters (Song et al., 2010; Westerholm et al., 2011, 2012). Pure cultures of *Methanoseta* spp. have been shown to be the most sensitive to ammonium stress (Karakashev et al., 2005; Steinhaus et al., 2007; Nettmann et al., 2010). *Methanoseta concilii*, for instance, showed an optimum NH$_4^+$-N concentration of < 1.1 g L$^{-1}$ and their growth reduced sharply at a concentration beyond 1.9 g NH$_4^+$-N L$^{-1}$ at 35 °C (Steinhaus et al., 2007). On the other hand, the hydrogenotrophic *Methanomicrobiales* remained active at total ammonia-N levels exceeding 3 g L$^{-1}$ (Schnürer et al., 1999, Angenent et al., 2002; Nettmann et al., 2010; Westerholm et al., 2011, 2012). However, the increase of ammonium to 400 mM NH$_4^+$-N (7.2 g NH$_4^+$-N L$^{-1}$) stopped methane production from *Methanospirillum hungatei* (Sprott et al., 1985; Sprott & Patel, 1986), whereas the growth of *Methanobacterium* spp. remained unaffected (Sprott & Patel, 1986). The
aceticlastic *Methanosarcina* had also been shown to tolerate up to 7 g NH$_4^+$-N L$^{-1}$ ammonium (Westholm *et al.*, 2012; Fotidis *et al.*, 2013). In the sludge used in the present study, there was only a small amount of *Methano-
sarcinaceae* in the gene profiles, but the accumulation of acetate upon prolonging incubation under high ammonium concentrations probably activated *Methanosarcina* spp. that contributed to CH$_4$ production in the later stages. It has to be noted, however, that pH could significantly regulate the observed effects of ammonium stress. Caution should be taken when comparing digester slurries in different experiments with varying pH values.

In summary, our study demonstrated that the inhibition of methanogenesis in anaerobic digester sludge was largely due to the repression of functional gene transcription rather than the total population of methanogens. The composition of *mcrA* transcripts reacted strongly to ammonium stress, whereas the structure of the methanogen community was less affected.

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**References**


Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. The time-course of NH₃-N in sludge incubations with the treatments of 0, 3, 7, and 10 g NH₄⁺-N L⁻¹, respectively; data are mean = SD (n = 3).

Fig. S2. Box-chart analysis of logarithm-transformed data from Fig. 5 describing the relative abundance of mcrA copy numbers during the entire cultivation period. Different letters above the boxes indicate a significant difference (P < 0.01).