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Published in:
Bioresource Technology

Link to article, DOI:
[10.1016/j.biortech.2017.11.081](https://doi.org/10.1016/j.biortech.2017.11.081)

Publication date:
2018

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):

Tian, H., Fotidis, I., Kissas , K., & Angelidaki, I. (2018). Effect of different ammonia sources on acetoclastic and hydrogenotrophic methanogens. *Bioresource Technology*, 250, 390-397.
<https://doi.org/10.1016/j.biortech.2017.11.081>

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Accepted Manuscript

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PII: S0960-8524(17)32085-0
DOI: <https://doi.org/10.1016/j.biortech.2017.11.081>
Reference: BITE 19231

To appear in: *Bioresource Technology*

Received Date: 6 November 2017
Revised Date: 22 November 2017
Accepted Date: 23 November 2017

Please cite this article as: Tian, H., Fotidis, I.A., Kissas, K., Angelidaki, I., Effect of different ammonia sources on aceticlastic and hydrogenotrophic methanogens, *Bioresource Technology* (2017), doi: <https://doi.org/10.1016/j.biortech.2017.11.081>

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1 **Effect of different ammonia sources on acetoclastic and**
2 **hydrogenotrophic methanogens**

3

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13 **Abstract**

14 Ammonium chloride (NH₄Cl) was usually used as a model ammonia source to simulate
15 ammonia inhibition during anaerobic digestion (AD) of nitrogen-rich feedstocks. However,
16 ammonia in AD originates mainly from degradation of proteins, urea and nucleic acids, which
17 is distinct from NH₄Cl. Thus, in this study, the inhibitory effect of a “natural” ammonia
18 source (urea) and NH₄Cl, on four pure methanogenic strains (aceticlastic: *Methanosarcina*
19 *thermophila*, *Methanosarcina barkeri*; hydrogenotrophic: *Methanoculleus bourgensis*,
20 *Methanoculleus thermophilus*), was assessed under mesophilic (37°C) and thermophilic (55°C)
21 conditions. The results showed that urea hydrolysis increased pH significantly to unsuitable
22 levels for methanogenic growth, while NH₄Cl had a negligible effect on pH. After adjusting
23 initial pH to 7 and 8, urea was significantly stronger inhibitor with longer lag phases to
24 methanogenesis compared to NH₄Cl. Overall, urea seems to be more toxic on both aceticlastic
25 and hydrogenotrophic methanogens compared to NH₄Cl under the same total and free
26 ammonia levels.

28 **Keywords**

29 Ammonia inhibition; Ammonium chloride; Anaerobic digestion; Pure strain; Urea.

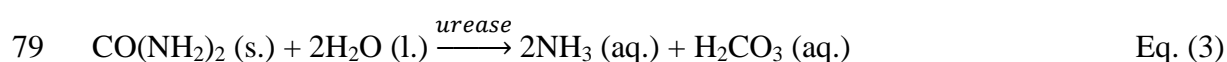
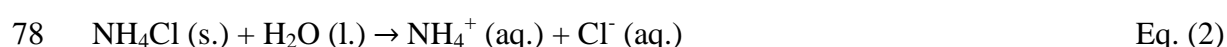
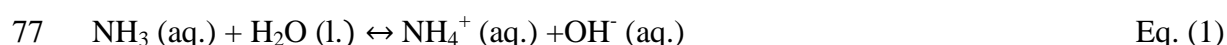
30 1 Introduction

31 Biogas (a mixture of CH₄ and CO₂) is an attractive renewable energy (Holm-Nielsen et al.,
32 2009), which is formed during anaerobic digestion (AD) of different biomasses. As one of the
33 most promising and widely used green technologies, AD is a complex biological process with
34 different microorganisms involved, which can reduce the waste pollution and offset part of the
35 energy usage (Chynoweth et al., 2001). However, it is reported that some potential substrates
36 are toxic to AD process by inhibiting the microorganisms' activity (Chen et al., 2008). Among
37 these substrates, nitrogen-rich substrates stand out, due to the ammonia formation during their
38 degradation. A low ammonia concentration (< 200 mg NH₄⁺-N L⁻¹) is beneficial to AD
39 process; nevertheless, relatively high ammonia levels (> 2000 mg NH₄⁺-N L⁻¹) would inhibit
40 AD, causing instability and even process failure (Liu and Sung, 2002). Total ammonia (TAN)
41 in aqueous solutions is the sum of ammonium ions (NH₄⁺) and free ammonia (FAN, NH₃).
42 The NH₄⁺ and NH₃ exist in an equilibrium (Eq. (1)), which is affected by the temperature and
43 the pH (Emerson et al., 1975). Specifically, FAN, which was suggested to be the most toxic
44 form of ammonia (Massé et al., 2014), increases along with temperature and pH.
45 Methanogenesis, the last step of AD process, is more sensitive to ammonia than hydrolysis,
46 acidogenesis and acetogenesis steps (Yenigün and Demirel, 2013). Furthermore, in most of
47 the studies, hydrogenotrophic methanogens were reported to be more robust to ammonia
48 toxicity than acetoclastic methanogens (Schnürer et al., 1999; Werner et al., 2014; Dai et al.,
49 2017). However, controversial results can also be found (Calli et al., 2005; Karakashev et al.,
50 2005).

51 Considering ammonia inhibition is such a serious and highly debated topic, a great
52 number of studies focusing on the impact of ammonia levels and on inhibition mechanism
53 have been conducted in different reactor types (Angelidaki and Ahring, 1993; Sung and Liu,
54 2003; Cuetos et al., 2008; Wang et al., 2015; Chen et al., 2016). As a result, it is reviewed that

55 50% inhibition was caused by TAN concentrations ranging from 1700 to 14000 mg NH₄⁺-N
 56 L⁻¹ depending on different experimental conditions (Chen et al., 2008). However, in most of
 57 the aforementioned studies, ammonium chloride (NH₄Cl) was used as the inhibitor (ammonia
 58 source), and only few experiments can be found using other ammonia sources (Sterling et al.,
 59 2001; Westerholm et al., 2012; Dai et al., 2017). As a salt, NH₄Cl can dissociate immediately
 60 after addition into aqueous solutions and release chloride anions and ammonium cations, as
 61 shown in Eq. (2). However, since chloride anions could also be a potential inhibitor to AD
 62 process (Riffat and Krongthamchat, 2006; Viana et al., 2012), it is difficult to differentiate if
 63 the inhibitory effect only comes from ammonia. Moreover, in the real AD applications, when
 64 nitrogen-rich substrates are used as feedstocks, ammonia is usually formed by the degradation
 65 of proteins, urea and nucleic acids (Rajagopal et al., 2013). Furthermore, urea is the main part
 66 of animal urine besides water; thus abounds in animal slurry (e.g. poultry, mink pig, cattle)
 67 and slaughterhouse wastewater (Møller et al., 2004). Without urease, which is the enzyme that
 68 catalyses urea hydrolysis, urea in aqueous solutions has a negligible reaction rate constant of
 69 $6.3 \times 10^{-9} \text{ s}^{-1}$ and a half-life of 3.5 years (Krajewska, 2009). However, urease can be
 70 synthesized by different microorganisms, including some bacteria involved in AD process,
 71 which can accelerate the hydrolysis of urea by nearly 10^{14} times faster than the uncatalysed
 72 decomposition (Ciurli et al., 1999). As shown in Eq. (3), the direct hydrolysed product of urea
 73 is the most toxic ammonia form (i.e. FAN) (Zimmer, 2000). In addition, hydrolysis of urea
 74 causes sudden pH increase, which could negatively affect the AD process (Mobley et al., 1995;
 75 Ciurli et al., 1999).

76



80

81 Thus, in order to separate the inhibition only caused by ammonia and simulate this
82 process closer to realistic conditions, urea was used as ammonia source in reactors fed with
83 cattle manure (Sterling et al., 2001). However, among the limited studies using urea as
84 ammonia source, nothing can be found about its effect on methanogens. Considering
85 methanogenesis is the most sensitive step of AD process (Chen et al., 2008), it is important to
86 understand the urea effect on different methanogens. In addition, to date, there are no studies
87 assessing simultaneously the effect of NH_4Cl and urea on methanogenic archaea.

88 Therefore, the main aim of the present study was to investigate the effect of two different
89 ammonia sources on four pure methanogenic strains (i.e. two acetoclastic and two
90 hydrogenotrophic), under mesophilic (37°C) and thermophilic (55°C) conditions. To fulfil this
91 aim, firstly, the effect on pH caused by the NH_4Cl dissociation and urea hydrolysis in AD
92 batch reactors was investigated. Secondly, under controlled pH conditions (i.e. 7 and 8), five
93 different TAN levels (i.e. ten different FAN levels) were applied on each pure methanogenic
94 strain to evaluate the effect of the two ammonia sources on the cultures, independently of the
95 pH.

96 **2 Materials and methods**

97 **2.1 Pure strains, ammonia sources and enzyme**

98 Four pure methanogenic strains (acetoclastic: *Methanosarcina thermophila* TM-1 DSM
99 No.1825 and *Methanosarcina barkeri* MS DSM No. 800; hydrogenotrophic: *Methanoculleus*
100 *thermophilus* CR-1 DSM No. 2373 and *Methanoculleus bourgensis* MS2^T DSM No. 3045)
101 were purchased from DSMZ GmbH Company and used throughout the study. *M. thermophila*
102 and *M. thermophilus* are thermophilic, while *M. barkeri* and *M. bourgensis* are mesophilic
103 methanogens. All the pure strains were cultivated in the specific growth media suggested by

104 DSMZ GmbH Company. Specifically, the growth media used were medium 120 (DSMZ,
105 2014a) for *M. thermophila*, medium 120a (DSMZ, 2014b) for *M. barkeri*, medium 141
106 (DSMZ, 2017) for *M. thermophilus*, and medium 332 (DSMZ, 2014c) for *M. bourgensis*. The
107 carbon sources that were used for each strain were: acetate and methanol for *M. thermophila*;
108 CO₂ for *M. thermophilus*; methanol for *M. barkeri*; and formate and CO₂ for *M. bourgensis*.
109 Ammonium chloride (Sigma-Aldrich, CAS no. 12125-02-9) and urea (Sigma-Aldrich,
110 CAS no. 57-13-6) were used as ammonia sources for the main experiment. Urease (Type IX,
111 Sigma-Aldrich, CAS no. 9002-13-5) from *Canavalia ensiformis* (jack bean) seeds was used as
112 enzyme to hydrolyse urea. A buffer solution consisted of 0.2 M sodium phosphate with pH
113 7.3 was prepared for the dissolution of the enzyme before use.

114 **2.2 Experimental setup**

115 Two batch experimental assays were performed in this study to investigate the effect of
116 different ammonia sources on pH fluctuation of the reactors (Assay I) and on the
117 methanogenic process efficiency (Assay II). Before the experiments started, the pure strains,
118 bought from DSMZ (DSMZ GmbH Company, Germany), were cultivated according to its
119 corresponding cultivation protocols (DSMZ, 2014c; DSMZ, 2014b; DSMZ, 2014a; DSMZ,
120 2017). After several (4-6) generations, the cultures were used as inocula in the two
121 experimental assays of the current study with a 20/80 (v/v) inoculum to medium ratio
122 throughout the experiment. Meanwhile, urease was added to all batch reactors regardless of
123 the ammonia source. Furthermore, all the experiments were conducted in triplicates.

124 **2.2.1 Assay I: Effect on pH**

125 All the pure strains were tested under different ammonia levels as depicted in Table 1.
126 Serum vials were used with 40 and 118 mL working and total volume, respectively. After
127 adding the corresponding medium, each vial was closed with butyl rubber stopper and sealed
128 with aluminium caps, then flushed with a mixture gas of N₂/CO₂ (80/20, v/v) to create anoxic

129 conditions and autoclaved to provide sterile conditions. Other solutions that could not be
130 autoclaved according to the instructions (NaHCO_3 , Na_2CO_3 , Vitamin, Methanol, L-cysteine-
131 $\text{HCl}\cdot\text{H}_2\text{O}$ and $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$) were introduced by using sterilized, 0.2 μm pore size, Minisart[®]
132 NML Syringe Filters (Sartorius Stedim Biotech GmbH, Germany) to avoid any contamination.
133 $\text{Na}_2\text{S}\cdot 9\text{H}_2\text{O}$ solution was added as a reducing agent after inoculation. In addition, pure H_2
134 (62.4 mL) and CO_2 (15.6 mL) were added in the headspace of the batch reactors of the
135 hydrogenotrophic strains. Afterwards, all the batch reactors were incubated at their
136 corresponding temperatures ($37\pm 1^\circ\text{C}$ for mesophilic and $55\pm 1^\circ\text{C}$ for thermophilic). The pH
137 was measured after the urea hydrolysis finished (approximately 20 hours after the incubation
138 stated based on preliminary hydrolysis test, and the details were provided in the E-supplement
139 file).

140 **2.2.2 Assay II: Effect on methanogenesis**

141 In this assay, two different ammonia sources with five different TAN and ten different
142 FAN levels (as shown in Table 2) were tested on all the methanogens. For all the strains,
143 serum vials with 40 mL working volume was used, while total volume of 245 mL was used
144 for *M. thermophila* and *M. thermophilus* cultivation, and total volume of 118 mL was used for
145 *M. barkeri* and *M. bourgensis*. The reactors were closed with rubber stoppers, sealed with
146 aluminium caps, and flushed with a mixture N_2/CO_2 gas (80/20, v/v) after the addition of
147 medium. All the reactors containing medium were autoclaved before inoculation. Chemical
148 solutions, which could not be autoclaved, were added through sterilized filters afterwards. In
149 addition, for hydrogenotrophic *M. thermophilus* and *M. barkeri*, H_2/CO_2 (80/20, v/v) mixture
150 gas was injected into the headspace of the reactor to form 1 bar overpressure. Furthermore, a
151 pH adjustment strategy (the details were provided in the E-supplement file) was performed to
152 ensure the same pH levels (7 and 8) for each individual experiment using 4 M HCl and/ or
153 NaOH solutions. Specifically, for reactors with NH_4Cl , where the dissociation happened

154 immediately, pH adjustment was performed before the incubation started. However, for
155 reactors containing urea and the hydrolysis happened slowly, the pH was adjusted several
156 times until the hydrolysis finished (the details were provided in the E-supplement file). Finally,
157 all the batch reactors were incubated in their corresponding temperatures ($37\pm 1^\circ\text{C}$ for
158 mesophilic and $55\pm 1^\circ\text{C}$ for thermophilic).

159 **2.3 Analytical methods**

160 Methane accumulation in the headspace of the batch reactors was determined by a gas
161 chromatographer (Trace 1310 GC-TCD, Thermo Fisher, Denmark) equipped with a
162 TracePLOT TG-BOND Q 26004–6030 column (30 m x 0.32 mm I.D., film thickness 10 μm)
163 (Thermo Fisher), and helium was used as carrier gas (Tian et al., 2017). The pH of each
164 reactor was measured with PHM99 LAB pH meter (Radiometer TM).

165 **2.4 Calculations and statistics**

166 **2.4.1 Free ammonia**

167 The free ammonia concentration was calculated based on the following equation (Siles et
168 al., 2010):

$$169 \quad \text{FAN} = \frac{\text{TAN}}{1 + \frac{10^{-\text{pH}}}{K_a}} \quad \text{Eq. (1)}$$

170 where K_a is the dissociation constant affected by temperature, which equals to 1.29×10^{-9}
171 and 3.91×10^{-9} in this study for mesophilic and thermophilic condition, respectively.

172 **2.4.2 Methane production inhibition**

173 The methane production inhibition was defined as the ratio of the difference between
174 theoretical and practical methane production divided by the maximum theoretical methane
175 production. Maximum theoretical production, for the different carbon sources in the medium,
176 was calculated according to Angelidaki et al. (2011) and it was 122, 373 and 525 $\text{mL CH}_4 \cdot \text{g}^{-1}$

177 VS for formate, acetate and methanol. Meanwhile, for the H₂/CO₂ mixture gas, it was
178 calculated based on that 1 mL CH₄ forms from 4 mL H₂ and 1 mL CO₂.

179 2.4.3 Maximum specific growth rate

180 Maximum specific growth rate (μ_{\max}) was calculated through the OriginLab program
181 (OriginLab Corporation, Northampton, Massachusetts) by calculating the slope of the linear
182 part of the semi-logarithmic graph of the methane production of the reactors versus time
183 (Gray et al., 2009).

184 2.4.4 Statistical analysis

185 The OriginLab program was used for statistical analyses and data plotting. One-way and
186 two-way ANOVA were used to evaluate the statistically differences ($p < 0.05$) of ammonia
187 inhibition under different parameters (e.g. different ammonia sources, ammonia levels and pH
188 levels). Single outliers test was applied to the triplicate measurements if needed.

189 3 Results and discussion

190 3.1 Impact on pH from two different ammonia sources

191 The impact of urea hydrolysis and NH₄Cl dissociation on pH was significantly different
192 ($p < 0.05$, Fig. 1). Specifically, after urea hydrolysis completed, except for the basic TAN
193 levels, the pH increased to around 9 for *M. thermophila*, *M. barkeri*, and *M. bourgensis*,
194 which was outside of the pH limits (6.5-8.5) for AD process (Lay et al., 1998). This increase
195 in pH after urea hydrolysis, was in agreement with a previous study (Udert et al., 2003) where
196 elevated pH was observed alongside the extent of urea hydrolysis. The pH of *M. thermophilus*
197 increased alongside the urea concentration, and it was about 8.5 at the highest TAN level
198 (5000 mg NH₄⁺-N·L⁻¹). This different performance of *M. thermophilus* from the other strains
199 could be explained by the stronger buffer capacity in *M. thermophilus* medium compared to
200 the other media due to the higher NaHCO₃ concentration. In contrast, NH₄Cl dissociation did

201 not have any significant effect on the pH of batch reactors, with a maximum pH drop of
202 approximately 0.3 units at the highest TAN levels ($10000 \text{ mg NH}_4^+ \cdot \text{N} \cdot \text{L}^{-1}$). Therefore, it
203 seems that NH_4Cl is not a representative ammonia source to simulate ammonia inhibition in
204 AD reactors because, contrary to urea, it does not have an analogous pH effect.

205 Meanwhile, it also can be seen that a medium with strong buffer capacity could mitigate
206 the effect of urea hydrolysis on pH (e.g. *M. thermophilus* case); thus, it is reasonable to
207 suspect that the pH of manure-based AD reactors (high buffer capacity) would not increase in
208 such a great extent. At the same time, without pH adjustment, the pure strains are not expected
209 to grow with urea (except in the basic TAN concentrations), due to the unfavourable pH levels
210 (> 8.5). Therefore, all the following methanogenesis batch experiments in assay II, were
211 designed with a pH adjustment strategy (adjust the initial pH level to 7 and 8, respectively) to
212 compare the effect of the two different ammonia sources on the pure methanogenic strains,
213 independently of the pH.

214 **3.2 Methanogenesis performance of different methanogens**

215 **3.2.1 Aceticlastic *M. thermophile* and *M. barkeri***

216 Urea had similar or significantly higher ($p < 0.05$) inhibitory effect on both aceticlastic
217 strains compared to NH_4Cl in the majority of the tested TAN levels. For example, NH_4Cl
218 inhibited the methane production of *M. thermophila* by 58% at $5000 \text{ mg NH}_4^+ \cdot \text{N} \cdot \text{L}^{-1}$ (pH=8);
219 at the same time, urea inhibited the same strain more than 90% at $5000 \text{ mg NH}_4^+ \cdot \text{N} \cdot \text{L}^{-1}$ for
220 pH=7 and at all TAN levels above $3000 \text{ mg NH}_4^+ \cdot \text{N} \cdot \text{L}^{-1}$ for pH=8 (Fig. 2a). The different
221 inhibition effects were also reflected on the longer lag phases at the same ammonia levels for
222 urea compared to NH_4Cl . To be specific, up to threefold longer lag phase periods were in urea
223 reactors compared to NH_4Cl reactors (Table 3). Furthermore, at lower FAN levels ($< 151 \text{ mg}$
224 $\text{NH}_3 \cdot \text{N} \cdot \text{L}^{-1}$), μ_{max} of *M. thermophila* was between $0.04\text{-}0.06 \text{ h}^{-1}$ for both urea and NH_4Cl
225 reactors coinciding with μ_{max} values reported before (Sowers et al., 1984; Mladenovska and

226 Ahring, 2000). However, NH_4Cl reactors had significantly higher μ_{\max} compared to urea
227 reactor for FAN levels above $151 \text{ mg NH}_3\text{-N}\cdot\text{L}^{-1}$, which indicates a stronger inhibitory effect
228 of urea (Fig. 2c).

229 *M. barkeri* was the most sensitive methanogenic strain to ammonia compared to all the
230 other tested strains. Almost 100% inhibition was observed at $64 (5000 \text{ mg NH}_4^+\text{-N}\cdot\text{L}^{-1}, \text{pH}=7)$
231 and $89 \text{ mg NH}_3\text{-N}\cdot\text{L}^{-1} (7000 \text{ mg NH}_4^+\text{-N}\cdot\text{L}^{-1}, \text{pH}=7)$ for reactors with urea and with NH_4Cl ,
232 respectively (Fig.2b). These results were in accordance to previous studies reporting 50%
233 inhibition of *M. barkeri* growth at $42 \text{ mg NH}_3\text{-N}\cdot\text{L}^{-1}$ and more than 95% inhibition at 88 mg
234 $\text{NH}_3\text{-N}\cdot\text{L}^{-1}$ (Sprott and Patel, 1986; Hajarnis and Ranade, 1993). However, although complete
235 inhibition occurred in most ammonia levels, for FAN levels lower than $64 \text{ mg NH}_3\text{-N}\cdot\text{L}^{-1}$,
236 where methanogenesis was observed, urea was clearly stronger inhibitor than NH_4Cl .
237 Furthermore, urea prolonged the lag phase up to fourfold compared to NH_4Cl (Table 3). Even
238 though *M. barkeri* was the most sensitive methanogenic strain tested in the present study, it
239 had the highest μ_{\max} of $0.11\text{-}0.12 \text{ h}^{-1}$ (optimal conditions), which decreased alongside with the
240 increase of ammonia levels (Fig. 2d). Similar specific growth rates ($0.10\text{-}0.14 \text{ h}^{-1}$) of *M.*
241 *barkeri* were reported by Jarrell et al. (1987) when TAN was below $1.4 \text{ NH}_4^+\text{-N}\cdot\text{L}^{-1}$, and more
242 than 50% reduction was detected around $4 \text{ NH}_4^+\text{-N}\cdot\text{L}^{-1}$. However, no significant difference
243 ($p>0.05$) of the μ_{\max} can be found between urea and NH_4Cl reactors.

244 3.2.2 Hydrogenotrophic *M. thermophilus* and *M. bourgensis*

245 Overall, hydrogenotrophic methanogens were, as expected (Werner et al., 2014), more
246 tolerant to NH_4Cl than the acetoclastic methanogens tested in the current study. Interestingly,
247 it was also found that hydrogenotrophic methanogens were more tolerant to urea than
248 acetoclastic methanogens. Nevertheless, similar to acetoclastic strains, urea also had a higher
249 inhibitory effect on the hydrogenotrophic methanogens compared to NH_4Cl . However, there
250 was an exception for *M. thermophilus* at low TAN levels ($< 3000 \text{ mg NH}_4^+\text{-N}\cdot\text{L}^{-1}$), where

251 NH_4Cl seemed to be more toxic than urea (Fig. 3a). The reasons might be firstly, the pH of
252 the urea reactors did not increase due to the strong buffer capacity of *M. thermophilus*
253 medium as discussed previously; Secondly, NH_4Cl reactors suffered higher toxicity than urea
254 reactors at the beginning because of the higher ammonia concentration from instant NH_4Cl
255 dissociation compared to from the gradual urea hydrolysis process. However, at higher TAN
256 levels ($> 3000 \text{ mg NH}_4^+-\text{N}\cdot\text{L}^{-1}$), urea inhibited *M. thermophilus* significantly stronger ($p<0.05$)
257 than NH_4Cl . All the *M. thermophilus* reactors had a lag phase smaller than 1.2 days (Table 4)
258 maintaining a μ_{max} between $0.03\text{-}0.04 \text{ h}^{-1}$ indicating that *M. thermophilus* was able to cope
259 with the strong ammonia toxicity. This was in agreement with Wang et al. (2015) reporting no
260 significant drop ($p>0.05$) on methane production at ammonia levels up to $7000 \text{ mg NH}_4^+-\text{N}\cdot\text{L}^{-1}$
261 ¹ for *M. thermophilus* with a μ_{max} around 0.03 h^{-1} .

262 *M. bourgensis* was the most ammonia tolerant methanogenic strain tested in the current
263 study, in which no more than 15% inhibition was observed, and independently of the
264 ammonia sources, ammonia levels and pH levels (Fig.3b). This high tolerance was expected
265 because *M. bourgensis* was reported (Fotidis et al., 2014) to thrive under high ammonia levels
266 ($5000 \text{ mg NH}_4^+-\text{N L}^{-1}$). Moreover, Westerholm et al. (2015) observed that *M. bourgensis* was
267 the dominant archaeon in AD reactors operated under high FAN levels ($900 \text{ mg NH}_3\text{-N}\cdot\text{L}^{-1}$),
268 and Wang et al. (2015) also demonstrated that *M. bourgensis* can work properly at TAN
269 levels up to $7000 \text{ mg NH}_4^+-\text{N}\cdot\text{L}^{-1}$. However, even with this tolerant methanogen, urea was
270 proven more toxic than NH_4Cl , resulting in lag phases up to ten days for TAN levels above
271 $5000 \text{ mg NH}_4^+-\text{N}\cdot\text{L}^{-1}$ (pH 8), compared to only two days lag phase for the NH_4Cl at the
272 highest TAN levels. The same trend was observed among the specific growth rates, with
273 significantly lower μ_{max} for the urea reactors compared to NH_4Cl reactors in majority of the
274 tested ammonia levels.

2753 **The ammonia sources and the inhibition mechanism**

276 In general, urea was a significantly stronger inhibitor than NH_4Cl (Table 5). This could be
277 explained by the different manners that urea and NH_4Cl introduce TAN and FAN into the
278 reactors. Specifically, NH_4Cl , as an easily soluble salt, can fully dissociate in aqueous phase
279 immediately after its addition and the direct dissociative products are ammonium ions (Eq. 2),
280 instead of the more toxic FAN form (Massé et al., 2014). On the contrary, urea, which is an
281 organic compound, can only be hydrolysed slowly with the presence of urease, and produce
282 directly FAN (Eq. (3)), which is the most toxic ammonia form (Zimmer, 2000). Therefore,
283 relatively high FAN levels develop instantly after urea hydrolysis, before the final
284 $\text{NH}_4^+ \rightleftharpoons \text{NH}_3$ equilibrium (Eq. 1) is established, driven by the pH and the temperature
285 (Emerson et al., 1975). Compared to low FAN levels after NH_4Cl dissociation, this
286 momentary exposure of the methanogenic cells to such high FAN concentrations after urea
287 hydrolysis, could have a greater impact in their metabolic activity. Furthermore, NH_4Cl
288 dissociation does not have a significant effect on the pH of the reactor and thus does not create
289 unfavourable pH conditions for the methanogens. On contrary, urea hydrolysis without pH
290 control could increase the pH of the reactor into unfavourable levels. Even though pH was
291 adjusted constantly in the current experiment, until the hydrolysis of urea was completed, it
292 was impossible to avoid a temporal pH increase during the urea hydrolysis period (details are
293 provided in the E-supplement file). Thus the combined effect of momentary high FAN
294 concentrations and pH increase, even for short time periods during the hydrolysis phase, is
295 proposed as the main mechanism for the stronger inhibitory effect of urea compared to NH_4Cl
296 on the pure methanogenic strains tested in this study.

297 **4 Conclusions**

298 The current study demonstrated that urea was significantly more toxic compared to NH_4Cl
299 during AD process. Furthermore, urea hydrolysis resulted in a great pH increase to

300 unfavourable levels for methanogenic growth. However, a high buffer capacity can mitigate
301 the pH increase and lower the ammonia toxicity from urea. Additionally, hydrogenotrophic
302 methanogens were more tolerant, not only to NH_4Cl but also to urea, compared to aceticlastic
303 methanogens. Finally, considering only pure strains were tested in this study, further studies
304 in a more complex environment of real AD digesters are still needed to analyse the inhibition
305 effect of urea.

306 **Appendix A. Supplementary material**

307 E-supplementary data for this work can be found in e-version of this paper online: Fig. S1.
308 Preliminary urea hydrolysis test at different ammonia and pH levels with/ without urease
309 under two different incubation temperatures, a) for thermophilic *M. thermophila* and b) for
310 mesophilic *M. bourgensis*. Fig. S2. pH adjustment strategies to 7 and 8 at different urea
311 concentrations for a) *M. thermophila*, b) *M. barkeri*, c) *M. thermophilus*, d) *M. bourgensis*

312 **Acknowledgements**

313 This work was supported by Energinet.dk under the project framework ForskEL
314 “MicrobStop NH_3 -Innovative bioaugmentation strategies to tackle ammonia inhibition in
315 anaerobic digestion process” (program no. 2015-12327). Hailin Tian would like to thank for
316 the financial support from China Scholarship Council, and Konstantinos Kissas thanks
317 Alexander S. Onassis Public Benefit Foundation for granting a scholarship. The authors thank
318 Hector Garcia for his technical support during the experiments.

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- 429
430
431

432 **Figure legends**

433 **Fig. 1.** pH value after the hydrolysis of the urea and the dissolution of the NH_4Cl at different
434 ammonia levels, a) *M. thermophila*, b) *M. barkeri*, c) *M. thermophilus*, d) *M. bourgensis*

435 **Fig. 2.** Final methane production inhibition and μ_{max} of *M. thermophila* and *M. barkeri* under
436 different ammonia sources, ammonia levels and pH levels, a) inhibition of *M.*

437 *thermophila*, b) inhibition of *M. barkeri*, c) μ_{max} of *M. thermophila*, d) μ_{max} of *M.*

438 *barkeri*.

439 **Fig. 3.** Final methane production inhibition and μ_{max} of *M. thermophilus* and *M. bourgensis*

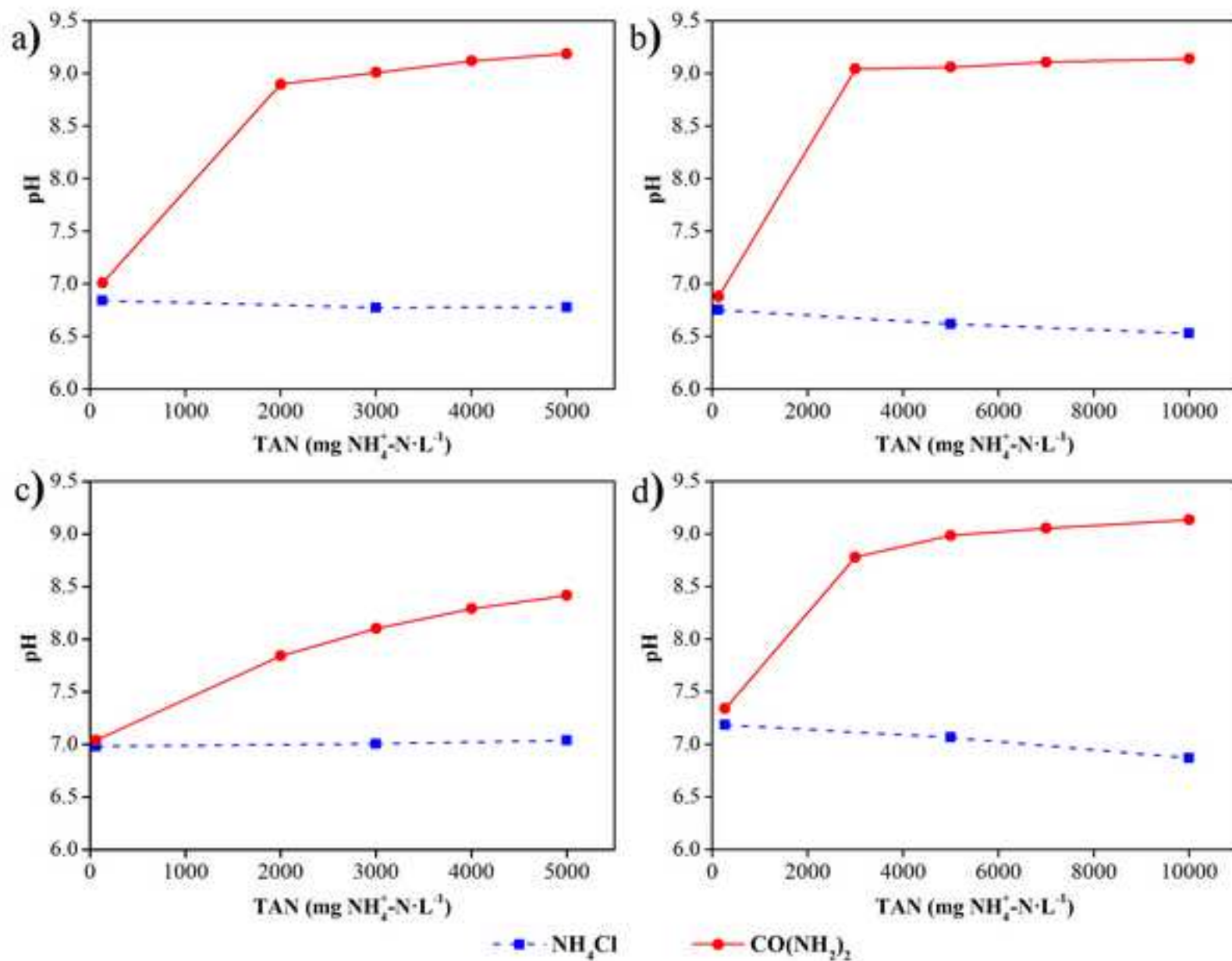
440 under different ammonia sources, ammonia levels and pH levels, a) inhibition of *M.*

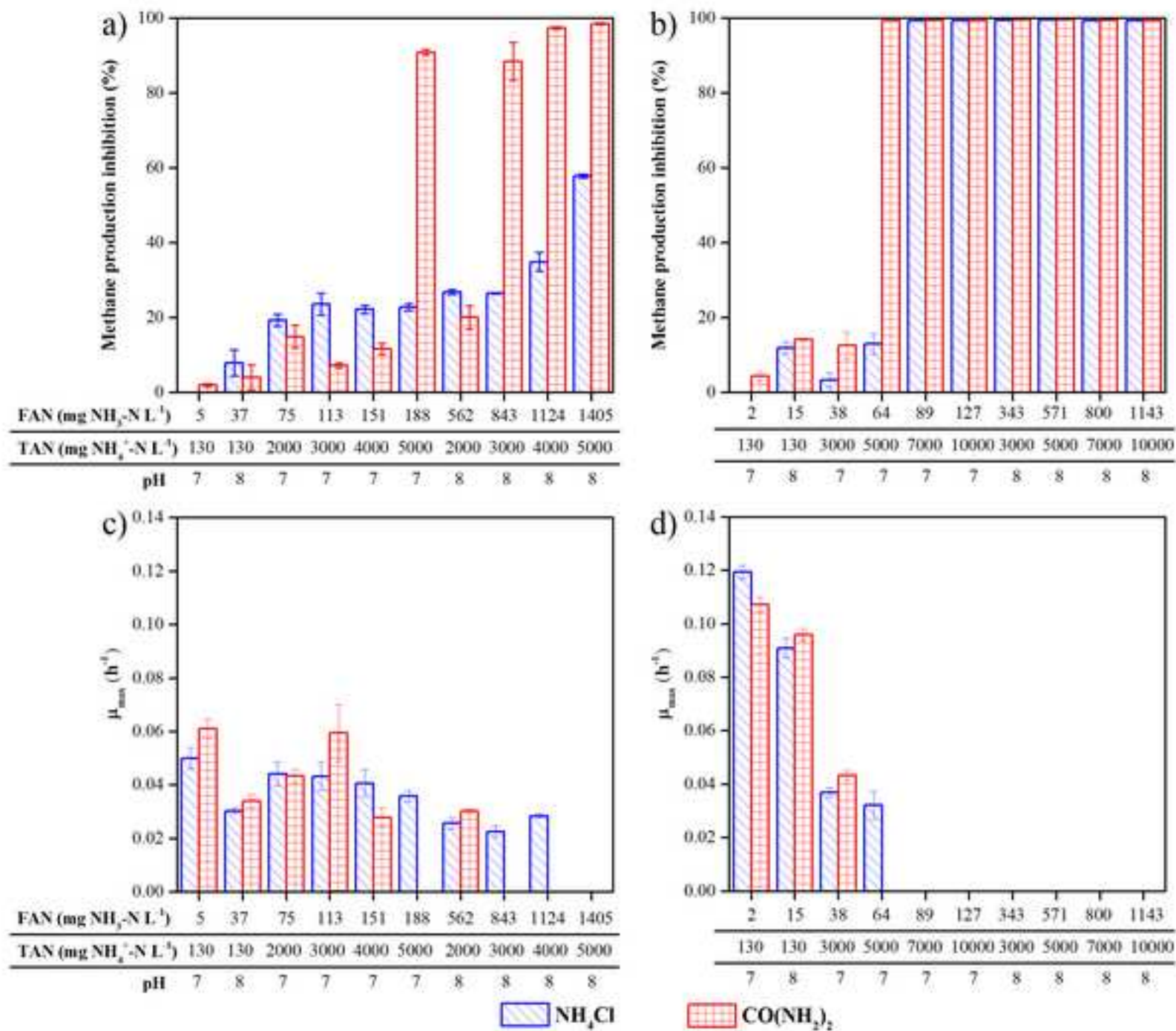
441 *thermophilus*, b) inhibition of *M. bourgensis*, c) μ_{max} of *M. thermophilus*, d) μ_{max} of *M.*

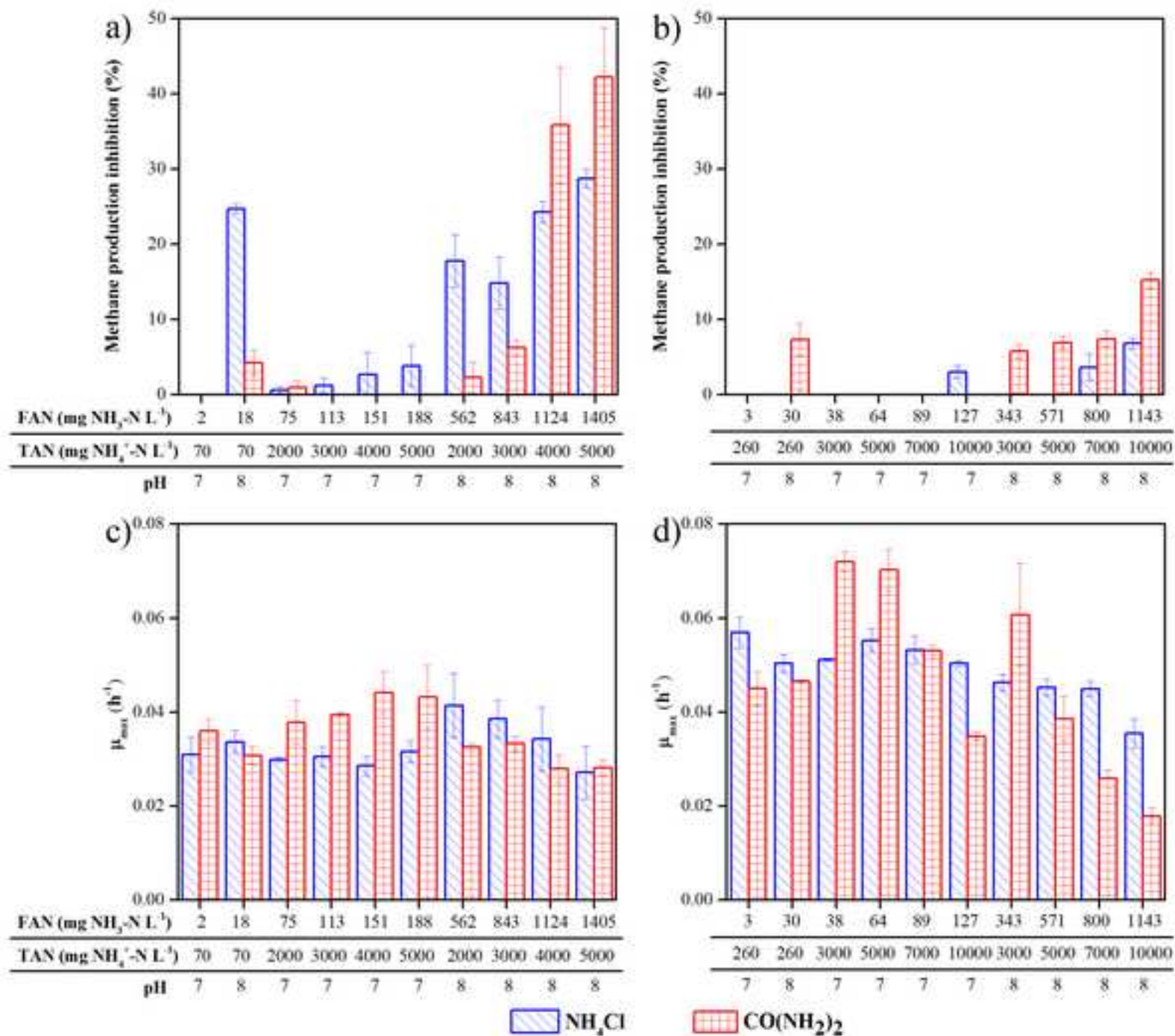
442 *bourgensis*.

443

444







445 **Table 1.** Different ammonia levels for the two ammonia sources in Assay I.

Strains	Ammonia sources	TAN (mg NH ₄ ⁺ -N·L ⁻¹) *
<i>M. thermophila</i>	CO(NH ₂) ₂	130, 2000, 3000, 4000 and 5000
	NH ₄ Cl	130, 3000 and 5000
<i>M. barkeri</i>	CO(NH ₂) ₂	130, 3000, 5000, 7000 and 10000
	NH ₄ Cl	130, 5000 and 10000
<i>M. thermophilus</i>	CO(NH ₂) ₂	70, 2000, 3000, 4000 and 5000
	NH ₄ Cl	70, 3000 and 5000
<i>M. bourgensis</i>	CO(NH ₂) ₂	260, 3000, 5000, 7000 and 10000
	NH ₄ Cl	260, 5000 and 10000

446 * The lowest TAN level is the basic ammonia levels of the medium.

447

448 **Table 2.** Different ammonia and pH levels under the two different ammonia sources of Assay
 449 II.

Strains	TAN (mg NH ₄ ⁺ -N·L ⁻¹) *	Ammonia sources	pH levels
<i>M. thermophila</i>	130, 2000, 3000, 4000 and 5000	NH ₄ Cl, CO(NH ₂) ₂	7, 8
<i>M. barkeri</i>	130, 3000, 5000, 7000 and 10000	NH ₄ Cl, CO(NH ₂) ₂	7, 8
<i>M. thermophilus</i>	70, 2000, 3000, 4000 and 5000	NH ₄ Cl, CO(NH ₂) ₂	7, 8
<i>M. bourgensis</i>	260, 3000, 5000, 7000 and 10000	NH ₄ Cl, CO(NH ₂) ₂	7, 8

450 * The lowest TAN level is the basic ammonia levels of the medium.

451

452 **Table 3.** Lag phase (days) of *M. thermophila* and *M. barkeri* under different experimental
 453 conditions.

Strains	Ammonia sources	pH	TAN levels (mg NH ₄ ⁺ -N·L ⁻¹)					
			130 (130) *	2000 (3000)	3000 (5000)	4000 (7000)	5000 (10000)	
<i>M. thermophila</i>	NH ₄ Cl	7	0	0	0	0	0	
		8	7.0 ± 3.0	11.0 ± 6.2	17.5 ± 7.5	32.6 ± 7.6	ND **	
	CO(NH ₂) ₂	7	0	0	3.6 ± 0.5	4.4 ± 0.5	ND	
		8	3.6 ± 1.9	33.0 ± 6.2	ND	ND	ND	
	<i>M. barkeri</i>	NH ₄ Cl	7	1.0	6.9	32.8 ± 5.9	ND	ND
			8	0.9	ND	ND	ND	ND
CO(NH ₂) ₂		7	1.1	24.8 ± 8.0	ND	ND	ND	
		8	1.2	ND	ND	ND	ND	

454 *Numbers outside parentheses were ammonia concentrations for *M. thermophila*, and the ones inside for *M.*
 455 *barkeri*.

456 ** ND: Not defined.

457

458

459 **Table 4.** Lag phase (days) of *M. thermophilus* and *M. bourgensis* under different experimental
 460 situation.

Strains	Ammonia sources	pH	TAN levels (mg NH ₄ ⁺ -N·L ⁻¹)				
			70 (260)*	2000 (3000)	3000 (5000)	4000 (7000)	5000 (10000)
<i>M. thermophilus</i>	NH ₄ Cl	7	0	0	0	0	0
		8	0	1.2 ± 0.5	1.2 ± 0.5	1.2 ± 0.8	0.9 ± 0.7
	CO(NH ₂) ₂	7	0	0	0	0	0
		8	0	0	0	0	0
<i>M. bourgensis</i>	NH ₄ Cl	7	0	0	0	0	0
		8	0	0	0	0	2.0
	CO(NH ₂) ₂	7	0	0	0	0	0
		8	0	1.0	2.7 ± 0.5	4.3	10.1

461 *Numbers outside parentheses were the ammonia concentrations for *M. thermophilus*, and the ones inside for *M.*
 462 *bourgensis*.

463

464 **Table 5.** Overall comparison of highest methane production inhibition of all strains.

Strains	pH	NH ₄ Cl	CO(NH ₂) ₂
<i>M. thermophila</i> *	7	22.9 ± 0.9 %	91.0 ± 0.8 %
	8	57.9 ± 0.5%	98.5 ± 0.2 %
<i>M. barkeri</i> **	7	99.4 ± 0 %	99.4 ± 0.1 %
	8	99.5 ± 0 %	99.6 ± 0.1 %
<i>M. thermophilus</i> *	7	3.8 ± 2.7 %	0%
	8	28.7 ± 1.2 %	42.2 ± 6.6 %
<i>M. bourgensis</i> *	7	3.1 ± 0.8 %	28.7 ± 1.2 %
	8	6.8 ± 0.7 %	15.2 ± 1.0 %

465 * Detected under the highest ammonia levels, specifically, for both pH levels, 5000 mg NH₄⁺-N·L⁻¹ for *M.*

466 *thermophila* and *M. thermophilus*, and 10000 mg NH₄⁺-N·L⁻¹ for *M. bourgensis*.

467 ** Detected under a relatively low ammonia levels, specifically, 7000 and 5000 mg NH₄⁺-N·L⁻¹ at pH 7 for

468 NH₄Cl and urea, respectively, and 3000 mg NH₄⁺-N·L⁻¹ at pH 8 for both.

469

470 **Highlights**

- 471 • Urea hydrolysis increases reactor pH significantly more than ammonium chloride
- 472 • Urea is more toxic to methanogenic archaea than ammonium chloride
- 473 • Combined high free ammonia and pH levels is the toxicity mechanism of urea
- 474 • Hydrogenotrophic methanogens are more robust than acetoclastic methanogens to urea

475

ACCEPTED MANUSCRIPT

