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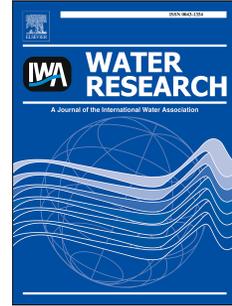
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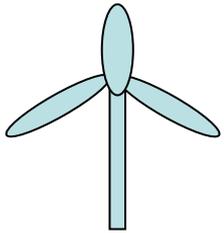
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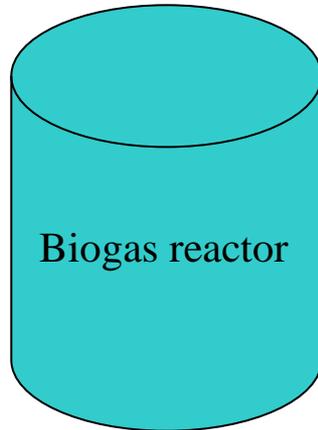
Water  
Electrolysis

Ammonia rich  
waste streams

$H_2$

A thick orange arrow pointing from the  $H_2$  box to the Biogas reactor.

A thick orange arrow pointing from the Ammonia rich waste streams box to the Biogas reactor.



Thermophilic/Mesophilic

Effect ???

A thick orange arrow pointing from the Biogas reactor to the Biomethanation box.

Biomethanation

A thick orange arrow pointing from the Biomethanation box to the Yield box.

Yield ✓

A thick orange arrow pointing from the Biomethanation box to the Biogas composition box.

Biogas  
composition ✗

1 Submission to Water Research

2 **Ammonia inhibition on hydrogen enriched anaerobic digestion of manure un-**  
3 **der mesophilic and thermophilic conditions**

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**24 Abstract**

25 Capturing of carbon dioxide by hydrogen derived from excess renewable energy  
26 (e.g., wind mills) to methane in a microbially catalyzed process offers an attractive  
27 technology for biogas production and upgrading. This bioconversion process is cat-  
28 alyzed by hydrogenotrophic methanogens, which are known to be sensitive to am-  
29 monia. In this study, the tolerance of the biogas process under supply of hydrogen,  
30 to ammonia toxicity was studied under mesophilic and thermophilic conditions.  
31 When the initial hydrogen partial pressure was 0.5 atm, the methane yield at high  
32 ammonia load ( $7 \text{ g NH}_4^+\text{-N L}^{-1}$ ) was 41.0% and 22.3% lower than that at low am-  
33 monia load ( $1 \text{ g NH}_4^+\text{-N L}^{-1}$ ) in mesophilic and thermophilic condition, respective-  
34 ly. Meanwhile no significant effect on the biogas composition was observed. More-  
35 over, we found that hydrogenotrophic methanogens were more tolerant to the ammo-  
36 nia toxicity than acetoclastic methanogens in the hydrogen enriched biogas produc-  
37 tion and upgrading processes. The highest methane production yield was achieved  
38 under 0.5 atm hydrogen partial pressure in batch reactors at all the tested ammonia  
39 levels. Furthermore, the thermophilic methanogens at 0.5 atm of hydrogen partial  
40 pressure were more tolerant to high ammonia levels ( $\geq 5 \text{ g NH}_4^+\text{-N L}^{-1}$ ), compared  
41 with mesophilic methanogens. The present study offers insight in developing re-  
42 sistant hydrogen enriched biogas production and upgrading processes treating am-  
43 monia-rich waste streams.

**44 Keywords**

45 Anaerobic digestion; Ammonia inhibition; Hydrogenotrophic methanogens; Hydro-  
46 gen; Wastewater treatment

## 47 1. Introduction

48 Anaerobic digestion (AD) is a sustainable technology that has been used for the  
49 treatment of various waste streams such as animal manure, food waste and sludge.  
50 However, AD treatment of the substrates containing high total ammonia (ammoni-  
51 um ion and free ammonia) concentration can be seriously inhibited by the ammonia  
52 which is produced during the biodegradation of proteins, urea and nucleic acids.  
53 There are two principal forms of inorganic ammonia nitrogen in aqueous solution:  
54 Ammonium ion ( $\text{NH}_4^+$ ) and free ammonia ( $\text{NH}_3$ ).  $\text{NH}_3$  has been considered to be  
55 the main inhibitor (Rajagopal et al., 2013; Yenigün & Demirel, 2013).  $\text{NH}_3$  mole-  
56 cules diffuse into the microbes' cells freely which can cause proton imbalance, in-  
57 crease maintenance energy requirements, change intracellular pH and inhibit specif-  
58 ic enzyme reactions (Gallert et al., 1998; Sprott & Patel, 1986).  $\text{NH}_3$  concentration  
59 mainly depends on temperature, pH and total ammonia concentration in anaerobic  
60 digestion process (Hafner & Bisogni, 2009). For example, the concentration of  $\text{NH}_3$   
61 increases with an increase in pH and/or temperature which causes the enhanced  
62 ammonia toxicity on the AD process (Nielsen & Angelidaki, 2008).

63 The AD process can be described by four distinctive steps namely: hydrolysis, aci-  
64 dogenesis, acetogenesis and methanogenesis. In detail, with the exception of the  
65 initial solubilisation of complex particulate material, methanogenesis seems to be  
66 the rate-limiting step. Moreover methanogens are the most vulnerable to ammonia  
67 compared to other groups of microorganisms involved in AD process (Angelidaki et  
68 al., 2011). There are two distinct methanogenic pathways for converting acetate to  
69 methane, which has been well described in previous studies (Fotidis et al., 2013;  
70 Wang et al., 2015). There are many papers referring on the sensitivity of the meth-

71 anogens to ammonia (Fotidis et al., 2013). It was reported that acetoclastic meth-  
72 anogens (i.e. *Methanosarcinaceae* spp. and *Methanosaetaceae* spp.) are more vul-  
73 nerable to ammonia toxicity compared to hydrogenotrophic methanogens (i.e.  
74 *Methanomicrobiales* spp., *Methanococcales* spp., *Methanocellales* spp., *Methano-*  
75 *bacteriales* spp. and *Methanopyrales* spp.) (Angelidaki & Ahring, 1993; Yenigün &  
76 Demirel, 2013).

77 Recently, an innovative AD process, which introduces hydrogen produced by water  
78 electrolysis using excess electricity from wind mill into anaerobic digester and sub-  
79 sequently converts it together with carbon dioxide in biogas into methane has been  
80 developed for simultaneous H<sub>2</sub> utilization and in-situ biogas upgrading (mainly re-  
81 fers to reduction of CO<sub>2</sub> content), giving synergistic advantages for both wind mills  
82 and biogas plants. (Deng & Hägg, 2010; Luo & Angelidaki, 2012; Luo et al., 2012).  
83 Such process has several advantages over conventional AD process: (1) low cost for  
84 further biogas upgrading since CO<sub>2</sub> content was reduced; (2) increase of methane  
85 production; (3) fully use of the wind mill capacity. Though promising, the H<sub>2</sub> en-  
86 riched AD process is just emerging from a technology perspective. There are sever-  
87 al challenges to be addressed for being able to develop a sustainable feasible tech-  
88 nology. One important aspect is the resistance of the process to ammonia inhibition,  
89 which is the very aspect that is unclear so far. Considering that most of the feed-  
90 stocks (e.g., cattle manure) in biogas plants (especially in Denmark) contain high  
91 level of ammonia, it is of outmost important to reveal the sensitivity of the process  
92 to high level of ammonia in order to accelerate the wide application of the technol-  
93 ogy. The outcome of such investigation will also help to find suitable strategy to  
94 counteract the ammonia inhibition.

95 During this process, enrichment of hydrogenotrophic methanogenic cultures in an-  
96 aerobic biogas reactors is occurring. In Luo and Angelidaki (2012)'s study, hydro-  
97 gen was injected into anaerobic reactors to achieve a hydrogen partial pressure of  
98 0.8 atm. After two months cultivation with H<sub>2</sub>, the hydrogenotrophic methanogenic  
99 activities increased to 198 mL CH<sub>4</sub> (g VSS h)<sup>-1</sup> under mesophilic and 320 mL CH<sub>4</sub>  
100 (g VSS h)<sup>-1</sup> under thermophilic condition, from around 10 mL CH<sub>4</sub> (g VSS h)<sup>-1</sup> of  
101 the original inoculum. This indicated that hydrogenotrophic methanogens were suc-  
102 cessfully enriched by long term injection of hydrogen. Thus, it would be obvious to  
103 assume that this process would be more resistant or tolerant to ammonia toxicity  
104 due to the enrichment of hydrogenotrophic methanogenesis compared to the con-  
105 ventional AD processes (Luo & Angelidaki, 2013b; Luo & Angelidaki, 2012; Luo  
106 et al., 2012). So far, information about the effect of ammonia toxicity on this inno-  
107 vative AD process is still lacking. Therefore, in this study, the effect of different  
108 ammonia levels on hydrogen enriched biogas upgrading process (different hydrogen  
109 partial pressure were included in the current study) in anaerobic reactors at both  
110 mesophilic and thermophilic temperature was explored.

## 111 **2. Materials and methods**

### 112 **2.1 Inoculum and feedstock**

113 The mesophilic and thermophilic inoculum were obtained from mesophilic and  
114 thermophilic anaerobic reactors in Hashøj Biogas plant (Denmark) and Snertinge  
115 Biogas Plant (Denmark), respectively. Both biogas plants use a mixture of manure  
116 (pig and cattle) and organic waste (fat and flotation sludge from food industries) as  
117 feedstock. As feedstock, dairy manure taken from Hashøj municipality (Denmark)  
118 was used in this study. The dairy manure was mixed in one plastic barrel and was

119 sieved, in order to remove the large solid particles, and then kept at -18 °C. Before  
120 use as substrate in the batch experiment, the frozen manure was thawed and stored  
121 at 4°C for 2-3 days. The basic characteristics of the inoculum and feedstock were  
122 analyzed and shown in Table 1.

123 **Table 1 is here**

## 124 **2.2 Experimental setup**

125 Both mesophilic and thermophilic inocula were incubated under four different am-  
126 monia concentrations (1, 3, 5 and 7 g NH<sub>4</sub><sup>+</sup>-N L<sup>-1</sup>) with NH<sub>4</sub>Cl as ammonia source.  
127 As batch reactors, vials with 118 mL total and 40 mL working volume, respectively  
128 were used. The working volume contained 10 mL inoculum, 10 mL dairy manure  
129 and 20 mL distilled water. After filling the content into the vials, butyl rubber stop-  
130 pers and aluminum crimps were used to seal them. Then all the batch reactors were  
131 flushed with nitrogen (flow rate 290 ml/s) for 10 min. Before the hydrogen injec-  
132 tion, the same volumes as the injected hydrogen of gas were extracted from the  
133 batch reactors to make sure the total pressure of all the batch reactors was the same.  
134 After that, 19.5, 39 and 78 mL of hydrogen were introduced with syringes into  
135 batch reactors to obtain different hydrogen partial pressure (0.25, 0.5, and 1 atm)  
136 for each ammonia level. Moreover, batch reactors without hydrogen addition, were  
137 also included. Additionally, reactors only with inoculum were used as blanks to  
138 evaluate the residual methane production. Two shaking incubators (37±1 °C and  
139 55±1°C, 180 rpm) were used for mesophilic and thermophilic batch reactors respec-  
140 tively and each condition was evaluated in triplicates (n=3).

### 141 2.3 Analytical methods

142 Total solids (TS), volatile solids (VS), pH, total ammonia and total Kjeldahl nitro-  
143 gen (TKN) were measured according to APHA's Standard Methods (Federation &  
144 Association, 2005). The pH level of the batch reactors was determined by using  
145 PHM99 LAB pH meter which was connected to the Gel pH electrode (pHC3105-8,  
146 Radiometer analytical). The electrode was filled with a gel containing KCl. Before  
147 measuring samples, the pH meter was calibrated at the temperature of the corre-  
148 sponding batch reactors. Shimadzu-14A gas chromatograph (GC) equipped with a  
149 thermal FID detector with hydrogen as a carrier gas (Shimadzu, Kyoto, Japan) was  
150 used to measure methane accumulation in the headspace of batch reactors. Hydro-  
151 gen concentration in batch reactors was measured by using GC-TCD fitted with a  
152 4.5 m×3 mms-m stainless column packed with Molsieve SA (10/80). Moreover, a  
153 gas-chromatograph (GCTCD) equipped with a column of 1.1 m × 3/16 "Molsieve  
154 137 and 0.7 m × 1/4" chromosorb 108 (MGC 82-12, Mikrolab A/S, Denmark) was  
155 used to determine the biogas composition in the headspace of batch reactors. The  
156 bottles were not vented during the whole experiment. The methane concentration  
157 (in percentage) in the headspace was measured by GC with pressure. Thus, the ac-  
158 cumulated methane was obtained by multiplying headspace volume of the batch  
159 reactors (78 ml) and the methane concentrations measured by GC. Additionally, the  
160 accumulated volatile fatty acids (VFA) concentration of the batch reactors were  
161 determined by using a gas-chromatograph (HP5890 series II) equipped with a flame  
162 ionization detector and a FFAP fused silica capillary column, (30 m × 0.53 mm i.d.,  
163 film thickness 1.5 µm), which uses nitrogen as carrier gas.

## 164 2.4 Calculations

### 165 2.4.1 Calculation of methane production

166 The hydrogen injected into the batch reactors was consumed by hydrogenotrophic  
167 methanogens to produce methane. Thus, the reactors with hydrogen addition had  
168 higher average methane yield compared to the reactors without hydrogen injection.  
169 Therefore, the calculation of subtracting the theoretical methane production from  
170 the introduced hydrogen in the batch reactors was made.

### 171 2.4.2 Statistical analysis

172 OriginLab program (OriginLab Corporation, Northampton, Massachusetts) was  
173 used for all the statistical analyses. For statistical analysis, one way Analysis of  
174 Variance (ANOVA) at 0.05 level was used. The effects of two factors (ammonia  
175 concentrations and hydrogen pressure) on methane production rate, methane pro-  
176 duction yield, VFA, pH level and carbon dioxide content were analyzed. All values  
177 presented are the means of independent triplicates (n=3)±SD.

## 178 3. Results and discussion

### 179 3.1 Accumulated methane yield of the reactors

180 In general, the methane yield decreased significantly ( $p < 0.05$ , P was ranging from  
181  $4.4 \times 10^{-8}$  to  $1.3 \times 10^{-5}$ ) with the increase of ammonia levels under all different hydro-  
182 gen partial pressures tested (Figure 1a). In detail, for the reactors without hydrogen  
183 injection, when ammonia concentration increased from 1 to 7 g  $\text{NH}_4^+\text{-N L}^{-1}$ , a de-  
184 crease of 65.0% in the methane yield was observed at mesophilic condition. For the  
185 mesophilic reactors adding hydrogen (0.25, 0.5 and 1 atm), inhibition caused by  
186 increasing ammonia level was also detected. However, the inhibition was less pro-  
187 nounced when  $\text{H}_2$  was added. More specifically, the methane yields at ammonia

188 level of  $7 \text{ g NH}_4^+\text{-N L}^{-1}$  were 42.7%, 41.0% and 48.3% lower compared  $1 \text{ g NH}_4^+\text{-N}$   
189  $\text{L}^{-1}$  for hydrogen additions of 0.25, 0.5 and 1 atm respectively (Figure 1a).

190

**Figure 1 is here**

191 Similarly, at thermophilic condition, the methane yield decreased by 44.2% in the  
192 reactors without hydrogen injection, when ammonia was increased from 1 to 7 g  
193  $\text{NH}_4^+\text{-N L}^{-1}$  (Figure 1a). Likewise the mesophilic conditions, inhibition was also  
194 less serious for the reactors with hydrogen. In addition, the highest methane yield  
195 at ammonia concentration of  $7 \text{ g NH}_4^+\text{-N L}^{-1}$  was observed under 0.5 atm initial  
196 hydrogen partial pressure both at mesophilic and thermophilic conditions. An inter-  
197 esting observation was that the methane yield in the thermophilic reactor was high-  
198 er than that in the mesophilic reactors with ammonia concentration of  $7 \text{ g NH}_4^+\text{-N}$   
199  $\text{L}^{-1}$  regardless of the initial hydrogen partial pressure. This is in particular noticea-  
200 ble as thermophilic methanogenesis is in general considered more ammonia sensi-  
201 tive.

202 At high ammonia concentration ( $7 \text{ g NH}_4^+\text{-N L}^{-1}$ ) even after subtracting the theoret-  
203 ical methane production from the introduced hydrogen (which was completely con-  
204 sumed in all the reactors) in the batch reactors higher methane production was ob-  
205 served, indicating that the tolerance to ammonia toxicity was promoted by hydrogen  
206 addition. Therefore, the results confirmed that the hydrogen enriched biogas up-  
207 grading process was more resistant to high ammonia levels compared to the con-  
208 ventional AD processes.

209 Ammonia is considered as an inhibitor of slowing down the growth and metabolic  
210 rates, therefore we calculated the methane production rates at different initial hy-  
211 drogen partial pressures (0, 0.25, 0.5 and 1 atm) under 1 and  $7 \text{ g NH}_4^+\text{-N L}^{-1}$  in

212 mesophilic and thermophilic conditions (Figure 1b). The length of time for calculat-  
213 ing methane production rate was from the beginning to the day that stable accumu-  
214 lated methane production was obtained (26 days, the whole length of the process  
215 was 48 days). The same tendency as for the methane yields, were shown for the  
216 methane production rates with ammonia concentration increase. In detail, the most  
217 serious inhibition occurred in the reactors without hydrogen injection at 7 g NH<sub>4</sub><sup>+</sup>-N  
218 L<sup>-1</sup> both in mesophilic (56.7% lower) and thermophilic (53.4% lower) conditions,  
219 which was in agreement with the methane yield result. Furthermore, at 7 g NH<sub>4</sub><sup>+</sup>-N  
220 L<sup>-1</sup>, the highest methane production rate was also achieved under 0.5 atm initial  
221 hydrogen partial pressure both at mesophilic (7.7 mL CH<sub>4</sub> (L • h)<sup>-1</sup>) and thermo-  
222 philic (13.4 mL CH<sub>4</sub> (L • h)<sup>-1</sup>) conditions.

223 **Figure 2 is here**

224 In general, the methane yield decreased with the increase of ammonia levels (Figure  
225 2). In detail, when ammonia concentration was increased to 5 and 7 g NH<sub>4</sub><sup>+</sup>-N L<sup>-1</sup>,  
226 the accumulated methane yield decreased significantly ( $p < 0.05$ ,  $p = 8.7 \times 10^{-7}$ ) in  
227 mesophilic condition. In thermophilic condition, the methane yield was affected  
228 less by the increasing ammonia levels compared to the mesophilic reactors.  
229 Both the results of methane yield and production rate indicated that the hydrogen  
230 based biogas upgrading process can still function at high ammonia level and was  
231 more tolerant compared to the conventional AD processes, though ammonia inhibi-  
232 tion occurred. The highest methane production yield was achieved under 0.5 atm  
233 hydrogen partial pressure in batch reactors at high ammonia levels. However, intro-  
234 ducing hydrogen to anaerobic biogas reactors could also lead to negative effect at  
235 least at the initial phase, until the hydrogen consumption rate by hydrogenotrophic

236 methanogens is equal or greater compared with the hydrogen production and injec-  
237 tion rate which may make a balance process again (Luo & Angelidaki, 2013a).  
238 Based on theoretical considerations but also by experimental proof, the increase of  
239 the hydrogen partial pressure in biogas reactors could cause decreased degradation  
240 of VFA, leading to process disturbance or break down (Fukuzaki et al., 1990; Luo  
241 et al., 2012; Siriwongrungron et al., 2007). Thus, the relatively lower methane yield  
242 and methane production rate at 1 atm (compared with 0.5 atm) indicated that the  
243 threshold of hydrogen partial pressure could be between 0.5 and 1 atm for causing  
244 disturbance of the process in the current study. Furthermore, an interesting observa-  
245 tion was that thermophilic batch reactors were more resistant under high ammonia  
246 levels (5 and 7 g  $\text{NH}_4^+\text{-N L}^{-1}$ ), compared with mesophilic reactors (0.5 atm). Free  
247 ammonia ( $\text{NH}_3$ ) has been considered to be the main toxic compound causing am-  
248 monia inhibition and high temperature will increase the free ammonia levels.  
249 Therefore, the result of the current study was contradictory to some previous stud-  
250 ies which reported that mesophilic methanogenesis is more resistant to high ammo-  
251 nia loads compared to the thermophilic process due to the lower free ammonia con-  
252 centrations (Chen et al., 2008; Fotidis et al., 2013). However, it was also (Wang et  
253 al., 2015a) previously reported that hydrogenotrophic thermophilic methanogens  
254 can tolerate higher ammonia and free ammonia concentrations compared to meso-  
255 philic methanogens, which was in agreement with the result of this study. Moreo-  
256 ver, in a previous study, thermophilic hydrogenotrophic methanogenic enrichment  
257 cultures were shown to be more efficient for methane production (122 mL  $\text{CH}_4$  (g  
258 VSS h)<sup>-1</sup> higher) compared to mesophilic enrichment cultures due to the higher  
259 rates of digestion, which could be another explanation (Luo & Angelidaki, 2012).

260 The discrepancy on the ammonia tolerance at mesophilic or thermophilic conditions  
261 could very well explained by the mechanism of ammonia inhibition. As it is as-  
262 sumed that free ammonia concentration ( $\text{NH}_3$ ) is the active form for inhibition,  
263 which of course would constitute the thermophilic processes more susceptible for  
264 inhibition. However, this does not exclude the possibility that the thermophilic or-  
265 ganisms are more tolerant to free ammonia ( $\text{NH}_3$ ) levels. This could also be sup-  
266 ported by the evolutionary pressure in thermophiles to develop tolerance to free  
267 ammonia levels.

268 The results of the current study that high ammonia concentration can inhibit the  
269 hydrogen enriched biogas production and upgrading processes by lowering the me-  
270 thane yield should be noticed especially when substrates containing high ammonia  
271 levels are used. Moreover, one of the challenges that the innovative AD process has  
272 is the increasing of pH due to the consumption of carbon dioxide, which subse-  
273 quently will increase the free ammonia concentration and enhance the ammonia  
274 inhibition. Therefore, some sustainable and practical methods for counteracting  
275 ammonia inhibition on such processes are needed in the future. Controlling pH lev-  
276 els by co-digestion with appropriate low pH substrates could be an optional solution.  
277 For example, in a previous study, Luo and Angelidaki (2013a) maintained the pH  
278 level in an optimal range for anaerobic digestion in the biogas reactor with addition  
279 of hydrogen by co-digestion of manure and acidic whey.

### 280 **3.2 VFA Accumulation and pH levels**

281 Generally, the total VFA concentrations of the reactors increased with the increas-  
282 ing ammonia levels. The reactors with initial hydrogen partial pressure of 0.5 atm  
283 had the lowest VFA concentrations, indicating a healthy AD process without VFA

284 accumulation and inhibition of methanogenesis, which was in agreement with the  
285 results of the methane yield (Figure 3). Specifically, under mesophilic condition  
286 and ammonia levels of  $7 \text{ g NH}_4^+\text{-N L}^{-1}$ , the VFA concentrations were 1.3 and 1.6 g  
287  $\text{L}^{-1}$  at 0 and 1 atm of hydrogen partial pressure respectively, which were significant-  
288 ly ( $p < 0.05$ ,  $p = 1.7 \times 10^{-8}$ ) higher compared with ones at 0.25 and 0.5 atm (Figure 3a).  
289 Additionally, at 0.5 atm of hydrogen partial pressure, total VFA at all tested ammo-  
290 nia levels were below  $0.4 \text{ g L}^{-1}$  (Figure 3a) and similar results were obtained under  
291 thermophilic condition.

292 **Figure 3 is here**

293 High hydrogen partial pressure is considered to cause inhibition of propionate and  
294 butyrate degradation (Fukuzaki et al., 1990; Siriwongrungson et al., 2007). Howev-  
295 er, at shaking speed of 100 rpm under 1 atm of hydrogen partial pressure, no inhibi-  
296 tion of either propionate or butyrate degradation was observed (Luo et al., 2012).  
297 The hydrogen's slow mass transfer from gas to the liquid phase combined with the  
298 fast consumption rate of the dissolved hydrogen by the hydrogenotrophic methano-  
299 gens, was the procedure for keeping dissolved hydrogen level low for efficient deg-  
300 radation of propionate and butyrate (Fukuzaki et al., 1990). On the contrary in the  
301 current study, the relatively higher shaking speed (180 rpm) applied may cause fast  
302 hydrogen transfer to the liquid phase resulting in more dissolved hydrogen in liq-  
303 uid, and along with the high ammonia level ( $7 \text{ g NH}_4^+\text{-N L}^{-1}$ ) could be the reason of  
304 the increase of the VFA concentrations at 1 atm of hydrogen partial pressure. On  
305 the contrary for the middle hydrogen partial pressures (0.25 and 0.5 atm), lower  
306 VFA concentrations were obtained. The reason for the less VFA accumulation at

307 0.25 and 0.5 atm could be the lower dissolved hydrogen level in the liquid and also  
308 the resistance to ammonia toxicity.

309 The pH levels in both mesophilic and thermophilic batch reactors were shown in  
310 Figure 4. At 0 and 1 atm of hydrogen partial pressure, the pH decreased from 7.95  
311 to around 7.80 ( $7 \text{ g NH}_4^+\text{-N L}^{-1}$ ), while at 0.5 atm, the pH levels under different  
312 ammonia concentrations increased from 7.95 to around 8.10. During anaerobic di-  
313 gestion of cattle manure, several substances such as ammonia, bicarbonate, and  
314 VFA could affect pH levels (Batstone et al., 2002). Therefore, at ammonia concen-  
315 tration of  $7 \text{ g NH}_4^+\text{-N L}^{-1}$ , the significant increase ( $p < 0.05$ ,  $p = 6.9 \times 10^{-5}$  at mesophilic,  
316  $p = 2 \times 10^{-5}$  at thermophilic) of pH at 0.25 and 0.5 atm was caused by the consumption  
317 of bicarbonate which was used by hydrogenotrophic methanogens for methane  
318 production (Luo & Angelidaki, 2013a; Mu et al., 2006). However, the relatively  
319 lower pH at 1 atm and  $7 \text{ g NH}_4^+\text{-N L}^{-1}$ , was due to the accumulation of VFA.

320 **Figure 4 is here**

### 321 **3.3 Biogas composition**

322 In the mesophilic reactors, the carbon dioxide content decreased with the increasing  
323 of hydrogen partial pressure at ammonia concentration of  $7 \text{ g NH}_4^+\text{-N L}^{-1}$  (Figure  
324 5a). Nevertheless, no further decrease was observed when hydrogen partial pressure  
325 was higher than 0.5 atm. It was consistent with previous observation in hydrogen  
326 enriched biogas production and upgrading process at low ammonia load ( $\leq 2 \text{ g}$   
327  $\text{NH}_4^+\text{-N L}^{-1}$ ) (Luo et al., 2012). Although similar trend was observed in the thermo-  
328 philic reactors for initial hydrogen partial pressures of 0 and 0.5 atm, the carbon  
329 dioxide content further decreased at higher hydrogen partial pressure (1 atm) (Fig-  
330 ure 5a). This could be due to the higher conversion rates and activity of the micro-

331 organisms at thermophilic temperature which permits a faster removal of the hy-  
332 drogen and avoids accumulation of VFA. The results again confirmed that the hy-  
333 drogen enriched biogas upgrading processes can still function at high ammonia  
334 concentration.

335 **Figure 5 is here**

336 Comparatively, with fixed hydrogen pressure, the ammonia concentration had no  
337 significant influence on carbon dioxide content, both in mesophilic and thermo-  
338 philic conditions ( $p > 0.05$ ,  $p = 0.135$  at mesophilic,  $p = 0.138$  at thermophilic) (Figure  
339 5b). In detail, the carbon dioxide content was 38.1% and 40.0% (mesophilic and  
340 thermophilic, respectively) at  $7 \text{ g NH}_4^+\text{-N L}^{-1}$  without adding hydrogen which was  
341 in accordance with previously reported (Lindeboom et al., 2012). Meanwhile the  
342 methane content was around 80% when hydrogen was added, as it was reacting  
343 with carbon dioxide to produce methane. According to Figure 1a and 1b, the me-  
344 thane yield had a significant ( $p < 0.05$ ) decreasing at high ammonia levels. On the  
345 contrary, at the same ammonia concentration ( $7 \text{ g NH}_4^+\text{-N L}^{-1}$ ) the methane content  
346 increased (or carbon dioxide content decreased) in the reactors with hydrogen injec-  
347 tion, which indicated that hydrogenotrophic methanogens might be more resistant to  
348 high ammonia levels compared to acetoclastic methanogens in the hydrogen en-  
349 riched biogas production and upgrading processes.

#### 350 4. Conclusions

351 The results of the current study indicated that high ammonia concentration can  
352 inhibit the hydrogen enriched biogas production and upgrading processes by  
353 lowering the methane yield. Nevertheless, the ammonia concentration had no  
354 significant effect on the biogas composition in such processes. It also implied that  
355 the hydrogen enriched production and upgrading processes was more tolerant to  
356 high ammonia concentrations compared with conventional AD process. Moreover,  
357 thermophilic methanogens seemed to perform better compared with mesophilic  
358 methanogens under high ammonia levels (5 and 7 g NH<sub>4</sub><sup>+</sup>-N L<sup>-1</sup>). The current study  
359 was the first time to quantify ammonia toxicity for the hydrogen enriched biogas  
360 production and upgrading processes. Therefore, some sustainable and practical  
361 methods for counteracting ammonia inhibition on such processes (e.g., pH control  
362 or co-digested with low pH substrate) are needed in the future.

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443 **Table and figure captions**

444 **Table 1.** Characteristics of the inoculum and the dairy manure.

445 **Figure 1.** Methane yield (a) and methane production rate (b) as a function of hy-  
446 drogen partial pressure.

447 **Figure 2.** Methane yield as a function of ammonia concentrations at hydrogen par-  
448 tial pressure of 0.5 atm.

449 **Figure 3.** Total VFA accumulation under different hydrogen partial pressure (a)  
450 and under different ammonia concentrations (b).

451 **Figure 4.** pH levels at different hydrogen partial pressure under 7 g  $\text{NH}_4^+\text{-N L}^{-1}$  (a)  
452 and pH under different ammonia concentrations at hydrogen partial pressure of 0.5  
453 atm (b).

454 **Figure 5.** Carbon dioxide content under different hydrogen partial pressure (a) and  
455 under different ammonia concentrations (b).

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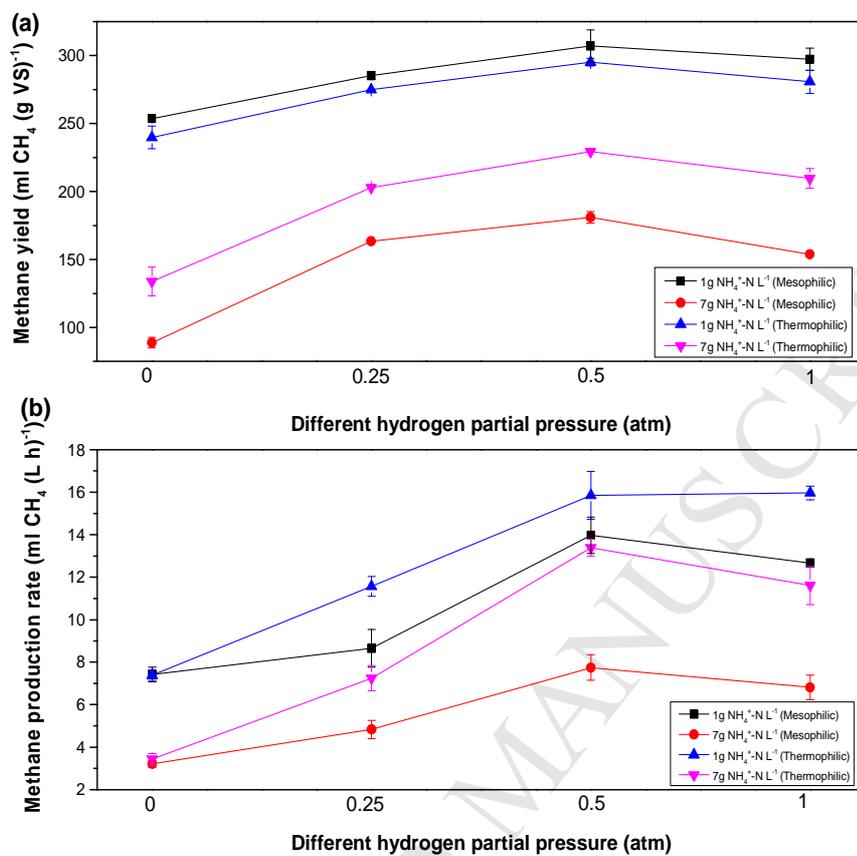
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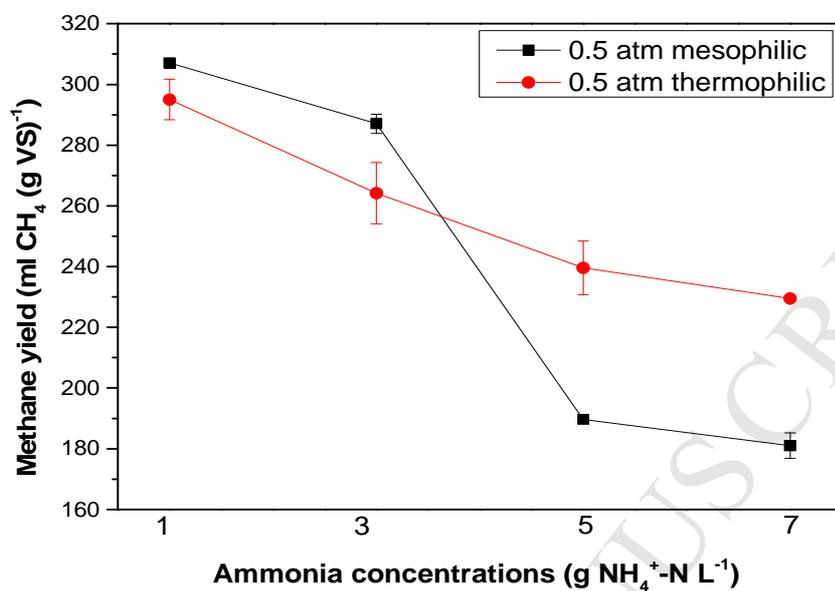
**Table 1.** Characteristics of the inoculum and the dairy manure

Parameter (unit)	Mesophilic Inoculum	Thermophilic Inoculum	Dairy manure
Density (g·L <sup>-1</sup> )	1003 ± 0.17	1003 ± 0.52	1002 ± 0.78
TS (g·L <sup>-1</sup> )	48.04 ± 0.24	31.24 ± 0.17	86.93 ± 0.00
VS (g·L <sup>-1</sup> )	28.42 ± 0.00	16.70 ± 0.00	63.30 ± 0.01
Total Kjeldahl nitrogen (g N L <sup>-1</sup> )	4.61 ± 0.21	4.23 ± 0.16	3.51 ± 0.13
Ammonia (g NH <sub>4</sub> <sup>+</sup> -N·L <sup>-1</sup> )	3.63 ± 0.09	3.04 ± 0.05	2.10 ± 0.08
Total VFA (mg L <sup>-1</sup> )	705.6±27.91	900.8 ± 24.40	3781 ± 137.14
pH	7.78	7.83	8.06

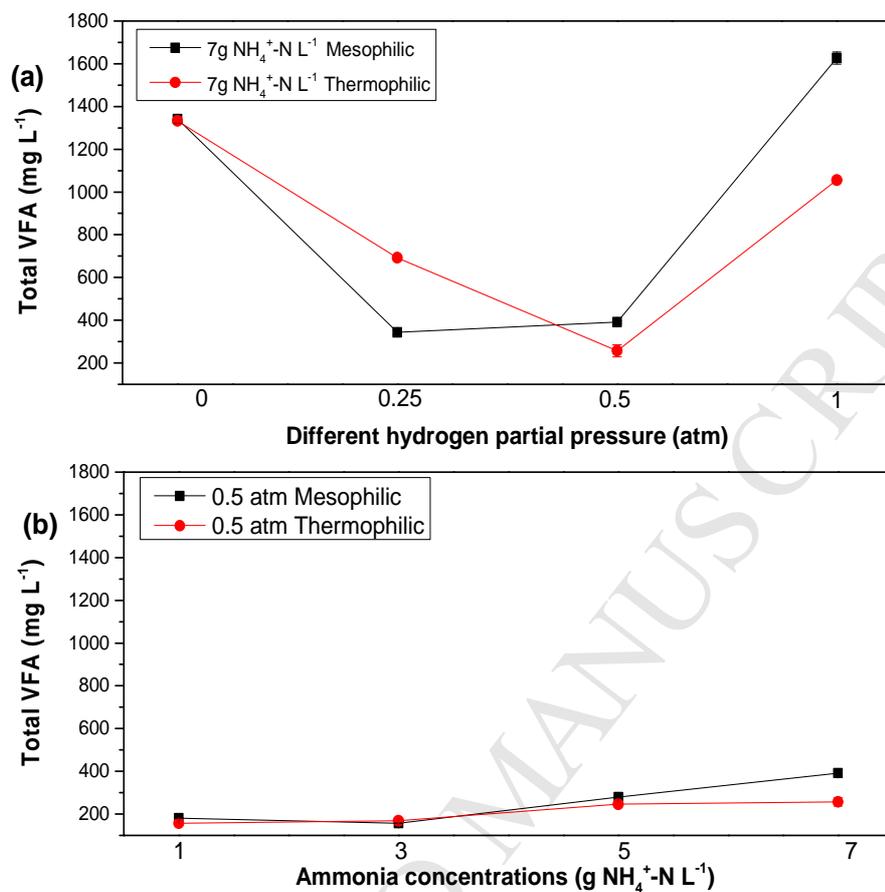
“±” means standard deviation and all values presented are the means of independent triplicates (n=3)



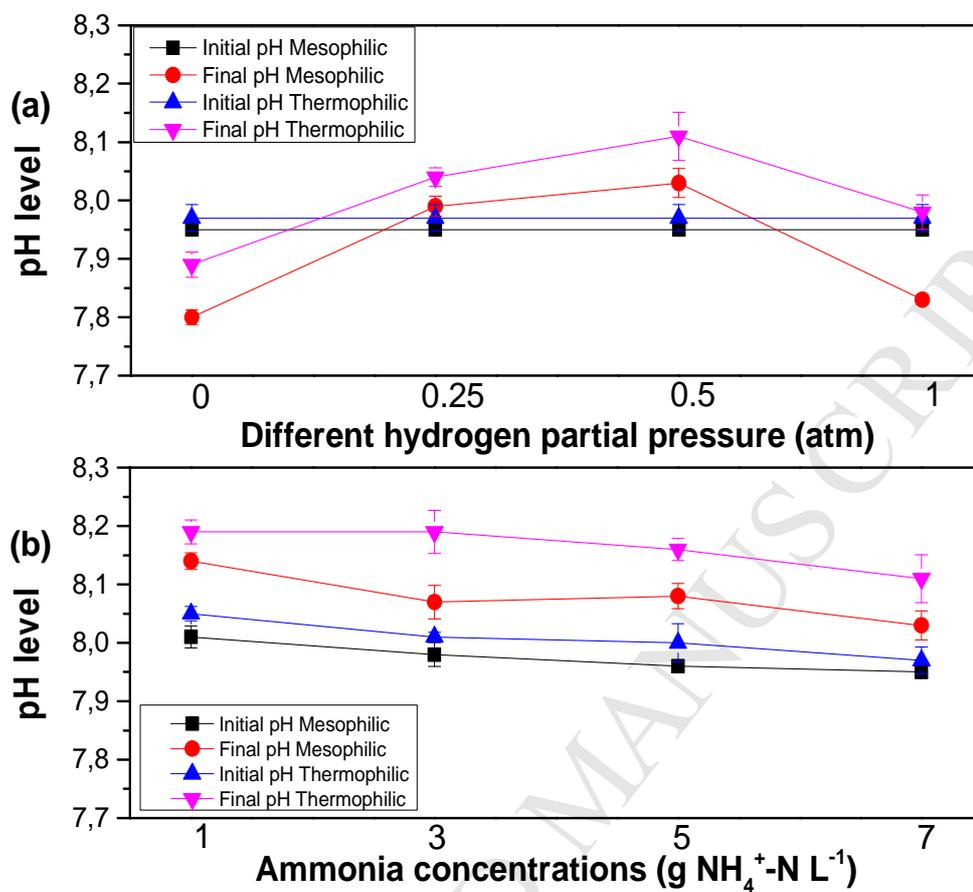
**Figure 1.** Methane yield (a) and methane production rate (b) as a function of hydrogen partial pressure.



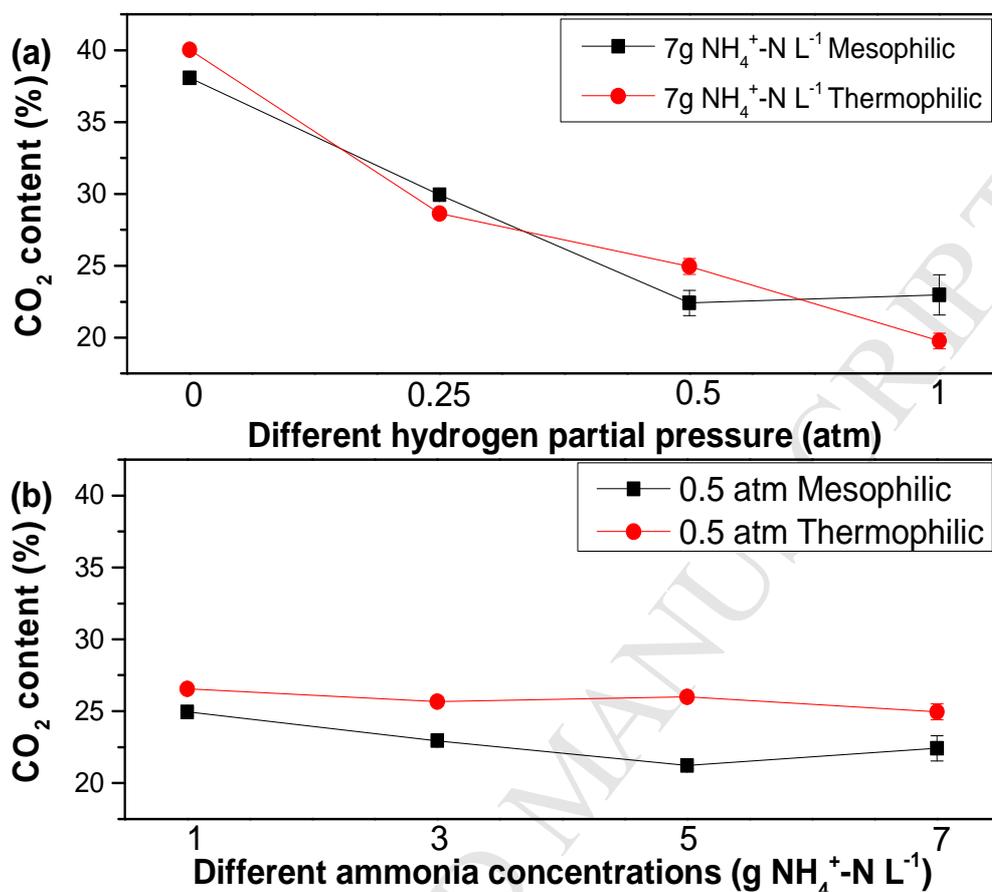
**Figure 2.** Methane yield as a function of ammonia concentrations at hydrogen partial pressure of 0.5 atm.



**Figure 3.** Total VFA accumulation under different hydrogen partial pressure (a) and under different ammonia concentrations (b).



**Figure 4.** pH levels at different hydrogen partial pressure under 7 g NH<sub>4</sub><sup>+</sup>-N L<sup>-1</sup> (a) and pH under different ammonia concentrations at hydrogen partial pressure of 0.5 atm (b).



**Figure 5.** Carbon dioxide content under different hydrogen partial pressure (a) and under different ammonia concentrations (b).

**Highlights**

- High ammonia concentration inhibited hydrogen enriched biogas upgrading processes.
- High ammonia concentration can lower the methane yield.
- The ammonia concentration had no significant effect on the biogas composition.
- Hydrogenotrophic archaea were more resistant to ammonia toxicity.
- The ammonia toxicity was alleviated at thermophilic condition.