Application of Response Surface Analysis to Evaluate the Effect of Concentrations of Ammonia and Propionic Acid on Acetate-Utilizing Methanogenesis

Seung Gu Shin 1, Joonyeob Lee 2, Trong Hoan Do 3, Su In Kim 2 and Seokhwan Hwang 2,*

1 Department of Energy Engineering, Future Convergence Technology Research Institute, Gyeongnam National University of Science and Technology, 33 Dongjin-ro, Jinju 52725, Gyeongnam, Korea
2 Division of Environmental Science and Engineering, Pohang University of Science and Technology, 77 Cheongam-ro, Nam-gu, Pohang 37673, Gyeongbuk, Korea
3 Vietnam Country Office, SEA Regional Program, 13th floor, HCMCC Tower, 249A Thuy Khue street, Tay Ho district, Hanoi 10000, Vietnam
* Correspondence: shwang@postech.ac.kr

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Abstract: Ammonia and propionate are known inhibitors of anaerobic methanogenesis at higher concentrations, and are likely to coexist in digesters treating high-strength wastewater. Therefore, this study was conducted to assess the effects of ammonia and propionate on acetate-utilizing methanogenesis when they coexist. Response surface analysis with face-centered central composite design was used to explore the total ammonia nitrogen (TAN) level of 2–5 g/L and the propionate level of 2–8 g/L in acetate-fed batch incubation. Two models were successfully derived to estimate the lag period and the methane yield in response to the concentrations of the two chemicals. The lag period was affected by both inhibitors, with clues obtained of a synergistic effect at a higher concentration range ([TAN] > 3.5 g/L and [propionate] > 5 g/L). The methane yield was also affected by the two inhibitors; between the two, it was more significantly dependent on the TAN concentration than on the propionate concentration. Real-time PCR showed that Methanosarcinaceae was the major methanogen group in this system. The results of this study improve our understanding of the inhibition of biogas reactors.

Keywords: anaerobic digestion; inhibition; response surface analysis; real-time PCR

1. Introduction

The instability of anaerobic system due to the presence of various inhibitors certainly limits the applicability of anaerobic digestion. Although ammonia (NH₃/NH₄⁺) is an important source of nitrogen for microbial growth in most environmental bioprocesses, a high level of its concentration has been considered as one of the major inhibitory effects associated in failure in numerous anaerobic digestions. Total ammonium-nitrogen (TAN) at a concentration in excess of 3.0 g/L, for example, is likely to be toxic to most anaerobes, especially methane producing bacteria (i.e., methanogens) [1]. This level of ammonia concentration is common in most agricultural wastewater, which has been subjected to anaerobic treatment [2]. An inhibitory mechanism by ammonia is primarily involved in passive transport of ammonia molecules across the cell membrane, resulting in disruption of proton motive force and/or homeostasis [3].

Propionic acid, one of the major volatile fatty acids (VFA) formed in acidogenesis of incoming organics to digestors, often accounts for up to 35% of the total methane produced in an anaerobic digestion [4]. This organic acid is not only one of the important major substrates in methanogenesis but the acid is also a major substance in high concentration that can cause instability of anaerobic digestion.
systems [5]. The specific mechanism of propionate toxicity, however, has rarely been understood compared to those by ammonia. Although the extent of inhibition by propionic acid is controversial, an unionized form of propionate is reported to be more toxic than the ionized one [6,7].

Methanogens, the methane-producing obligate anaerobes, comprise two archaeal groups: acetoclastic (i.e., acetate utilizers) and hydrogenotrophic (i.e., \( \text{H}_2/\text{CO}_2 \) utilizers). Because acetic acid is known to be a precursor for more than 70% of methane formation in most anaerobiosis [6], acetoclastic methanogens are believed to play a key role in stabilizing the pollution load of wastewater by participating in the terminal step, methanogenesis. The acetoclastic methanogens, however, are more sensitive to the presence of inhibitors such as ammonia and propionic acid than hydrogenotrophic methanogens [8,9].

Ammonia can originate from wastewater itself such as animal manure and the agricultural wastewater [10] and from the degradation of proteinaceous wastewaters including the fish- or meat-processing wastewaters [11]. Propionic acid mostly forms as a result of acidogenesis of organics in the wastewater [6]. When an anaerobic digester is inhibited by ammonia or other inhibitors, VFAs are likely to accumulate and propionic acid often persists longer than other VFAs due to unfavorable thermodynamics and necessity of syntrophism for degradation [12]. Therefore, it is evident that ammonia and propionate are likely to coexist in most anaerobic digestion, and higher levels of both compounds are expected when an incomplete methanation of VFAs occur initiated by ammonia inhibition. Thus, understanding the effects of the inhibitory substances on the acetoclastic methanation when they coexist is essential for an effective control of anaerobic digestion, especially for animal manure and agricultural waste management. Although an individual inhibitory threshold concentration of ammonia or propionic acid can be found in literature [3,13–17], information about simultaneous effects of the two potential inhibitors on acetoclastic methanation is lacking. Ammonia and propionic acid may impose opposite effects on pH when they penetrate into microbial cells; ammonia would increase the intracellular pH while propionic acid would decrease it. This means that the two inhibitors are likely antagonistic when they coexist. In contrast, synergistic inhibition effects caused by ammonia and some cations (i.e., \( \text{Na}^+ \), \( \text{K}^+ \), \( \text{Ca}^{2+} \), and \( \text{Mg}^{2+} \)) have been reported [3].

This study aimed to investigate the inhibitory effects of ammonia and propionic acid concentrations on acetoclastic methanogenesis when they coexist, in a systematic manner using response surface analysis (RSA) with a central composite design of experiment. The growth of acetoclastic methanogens was also quantified using real-time PCR for further discussion.

2. Material and Methods

2.1. Design of Experiment and Variables

RSA, a collection of mathematical and statistical techniques useful for analyzing the effects of several independent variables, is an iterative statistical technique to approximate multivariate responses [18] with the following equation:

\[
y = f(\sum_{i=1}^{n} x_i) + \varepsilon \quad (1)
\]

where, \( y \), \( x_i \), and \( \varepsilon \) denotes the response of the expected output; independent variable with \( i = 1, \ldots, n \) (\( n \) is the number of the independent variables); and the noise, respectively.

In this study, initial concentrations of TAN and propionate were the independent variables and designated as \( x_1 \) and \( x_2 \) (Equation (1)), respectively. Initial concentrations of each independent variable were adjusted to 2.0–5.0 NH\(_4\)-N/L and 2.0–8.0 g propionate/L. Ranges of the independent variables were carefully decided based on relevant literature reviews [3,8,15,19,20]. The design boundaries also fit the NH\(_4\)-N (up to 4.9 g/L) and propionate (up to 6.8 g/L) levels of full-scale anaerobic digesters in 24 biogas sites in a recent survey in Korea (data not shown). Two response variables were evaluated: the lag period and the methane yield.
A sequential procedure of collecting data, estimating polynomials with least squares method, and checking the adequacy of the model [21] was used to investigate the effects on methane production and variation in lag period of methanogenesis. Central composite design with a center point, replicated 3 times, was used. Minitab program (version 17.1.0) was used to analyze the data statistically [22].

2.2. Inoculum and Inhibition Test

Acetic acid (12 g/L) with growth nutrients was used for all subsequent experiments, including the inoculation system and the batch tests. Concentration of the nutrients in the substrate solution (i.e., mg/L) is as follows [23–25]: KH₂PO₄ 750, K₂HPO₄·3H₂O 1450, NaCl 600, KCl 370, NH₄Cl 816, MgSO₄·7H₂O 247, nitrilotriacetic acid 40, FeCl₃·6H₂O 4.89, MnCl₂·4H₂O 0.9, CoCl₂·6H₂O 1.5, CaCl₂·2H₂O 20, ZnCl₂ anhydrous 0.9, CuCl₂·2H₂O 4.3, H₃BO₃ 0.17, Na₂MoO₄·2H₂O 0.09, Na₂SeO₃·5H₂O 0.58, Na₂SO₄·10H₂O 260, NiCl₂·6H₂O 1.2, citric acid·H₂O 210. Anaerobic seed sludge from a local municipal wastewater treatment plant was cultivated in a lab-scale anaerobic continuously stirred tank reactor (CSTR) with 6.0-L working volume to produce constant inoculum, which would minimize any confounding effect associated with the use of inconsistent inoculum. The inoculum system was operated at 15 d hydraulic retention time at 35 °C, and the pH was maintained at 6.8 with 6.0 N NaOH and 3 N HCl, as described previously [26]. Methane production, residual acetate concentration, and volatile suspended solids (VSS) concentration in the effluent became steady after 60 d of operation.

Eleven batch trials for RSA were performed using identical anaerobic glass bottles (Schott Duran, Germany) with working volume of 0.5 L. In addition, one control trial was also prepared in the same manner but with no additional ammonia or propionate to the basal medium. Each bottle was equipped with a gas-tight bag to monitor and store the biogas produced. Ammonium solution (NH₄OH) and propionic acid (>99%) were respectively used to give desired initial TAN and propionate concentrations for each trial. Each bottle was seeded (approximately 40% v/v) using the effluent from the inoculum CSTR to give 200 ± 20 mg VSS/L. Initial pH was adjusted to 6.8 using 6 N NaOH solution and all bottles were incubated at 35 ± 0.5 °C. The bottles were purged with N₂ gas for 2 min at start-up to remove residual oxygen.

2.3. Analytical Methods

A gas chromatograph (6890 plus, Hewlett-Packard, CA), equipped with a HP Innowax capillary column and a flame ionization detector, was used to measure acetic and propionic acid concentrations. Helium was the carrier gas at a flow rate of 2.5 mL/min with a split ratio of 10:1. All samples were prepared by filtering through a 0.45 µm membrane filter. The filtered samples were acidified by injecting formic acid (1/200 volume) to convert the fatty acids to their unionized forms before analysis. Biogas production volume was measured using a gas-tight syringe. An identical gas chromatograph (6890 plus), equipped with a GS-Carbon Plot capillary column and a thermal conductivity detector, was used to measure the biogas composition, including methane.

2.4. Real-Time PCR

Total DNA was extracted from the seed inoculum and the RSA trials at the end of batch operation using a Magtration System 6GC (Precision System Science, Japan). The total archaea and the acetoclastic methanogens were quantified using real-time PCR with following primer-probe sets: Archaea (ARC), Methanosarcinaeae (Msc), and Methanosaetaceae (Mst) [27]. Real-time PCR analysis was performed using a LightCycler 1.2 system (Roche, Germany) as described previously [28].
3. Results

3.1. RSA Experiment

Twelve batch experiments, including the triplicate trials at the center and the control, were conducted following the face-centered central composite design (Table 1). The batch trials were operated for 60 d until no biogas was produced (Figure 1). The lag period of incubation (unit of d) and the methane yield (unit of L CH$_4$/g acetate fed) were estimated and used for the RSA analysis. Maximum methane production rate (unit of L/L/d) was also tested as a dependent variable (Table 1). However, models (1st to 2nd orders) derived from this parameter failed to pass the model validation criteria (adjusted $r^2 > 0.8$ and $p < 0.05$) and thus maximum methane production rate is not discussed further in this paper.

The acetate was initially fed at 12 g/L in all trials, but the residual acetic acid concentrations at the end of the batch incubation were dissimilar, accounting for 100% (control) to 46% (trial 3) consumption (Table 1). The end-of-batch pH was between 8.1 and 8.5, within a typical pH range (6.5–8.5) of anaerobic digestion.

![Figure 1. Profiles of methane production at different conditions. Blue, green and red colors represent initial TAN levels of 2.0 (N2), 3.5 (N3.5) and 5.0 (N5) g/L, respectively. Triangles (empty), circles (pale filled) and squares (filled) represent initial propionate levels of 2.0 (PA2), 5.0 (PA5) and 8.0 (PA8) g/L, respectively.](image-url)
Table 1. Experimental design and results of acetate-utilizing methanogenesis at different conditions.

| Trial Number | Initial $\text{NH}_4^+$-N Concentration (g/L) | Initial Propionate Concentration (g/L) | Estimated Lag Period (d) | Methane Yield (L $\text{CH}_4$/g acetate) | Maximum Methane Production rate (L/L/d) | Residual Acetate Concentration (g/L) | End pH |
|--------------|---------------------------------------------|--------------------------------------|--------------------------|------------------------------------------|----------------------------------------|-------------------------------------|--------|
|              |                                             |                                      | Observed                  | Predicted                                | Observed                               | Predicted                           |        |
| 1            | 2.0                                         | 2.0                                  | 0.8                       | 2.0                                      | 0.35                                   | 0.34                                | 0.73   | 8.4    |
| 2            | 2.0                                         | 8.0                                  | 8.7                       | 8.6                                      | 0.35                                   | 0.36                                | 0.39   | 0.58   | 8.4    |
| 3            | 5.0                                         | 8.0                                  | 28.0                      | 26.5                                     | 0.14                                   | 0.15                                | 0.12   | 6.48   | 8.1    |
| 4            | 5.0                                         | 2.0                                  | 11.0                      | 10.9                                     | 0.23                                   | 0.21                                | 0.29   | 4.11   | 8.2    |
| 5 **         | 3.5                                         | 5.0                                  | 8.0 (0.5)                 | 7.8                                      | 0.28 (0.2)                             | 0.28                                | 0.33 (0.02) | 2.17 (0.95) | 8.4 (0.1) |
| 6            | 2.0                                         | 5.0                                  | 4.5                       | 3.6                                      | 0.36                                   | 0.35                                | 0.48   | 0.63   | 8.5    |
| 7            | 3.5                                         | 8.0                                  | 13.5                      | 15.2                                     | 0.28                                   | 0.26                                | 0.30   | 2.71   | 8.3    |
| 8            | 5.0                                         | 5.0                                  | 15.2                      | 16.8                                     | 0.17                                   | 0.19                                | 0.16   | 0.08   | 8.3    |
| 9            | 3.5                                         | 2.0                                  | 5.3                       | 4.2                                      | 0.25                                   | 0.28                                | 0.32   | 3.35   | 8.3    |
| control      | 0.2                                         | 0.0                                  | 0.0 [9.4]                 | –                                        | 0.36                                   | –                                   | 0.47   | 0.00   | –      |

* The lag periods were subtracted by the lag period for the control (9.4 d). ** The experiment was replicated three times. Mean value (standard deviation).
3.1.1. Lag Period

As shown in Figure 1, a lag phase was observed in all trials. The control showed a lag period of 9.4 d, and this value was subtracted from the estimated lag periods of the other trials for further representation and discussion (Table 1). The shortest lag phase (10.2 d overall; 0.8 d subtracted by the control) was observed in trial 1, where the concentration of TAN and propionate were the lowest (2.0 g/L and 2.0 g/L, respectively). The longest lag phase (37.4 d overall; 28.0 d subtracted by the control) was observed in trial 3, which was introduced to the highest TAN (5.0 g/L) and propionate (8.0 g/L) concentrations.

After comparing the polynomial models of 1st to 2nd orders, a quadratic model was selected to fit the response of the lag period for the inhibition test:

\[
R_{LP} = 9.55 - 5.20X_1 - 2.03X_2 + 0.505X_1X_2 + 1.022X_1^2 + 0.211X_2^2
\]  

(2)

where, \(R_{LP}\) is the estimated response of the lag period, \(X_1\) is the TAN concentration, and \(X_2\) is the propionate concentration. This model fitted well with the observed data (adjusted \(R^2 = 0.95, p < 0.001\); Table 2) and no significant lack-of-fit was detected (\(p > 0.05\)). This indicates a good agreement between the experimental and the predicted data (Table 1). The residual plot confirmed that there was no significant pattern with regard to the fitted values (data not shown). Thus, the model (Equation (2)) is suggested to be able to accurately estimate the response surface within the study region.

| Term    | Coefficient | df * | F-value | p-Value |
|---------|-------------|------|---------|---------|
| Constant| 9.55        | –    | –       | –       |
| \(X_1\) | –5.20       | 1    | 111.54  | 0.000   |
| \(X_2\) | –2.03       | 1    | 81.85   | 0.000   |
| \(X_1X_2\) | 0.505     | 1    | 8.31    | 0.034   |
| \(X_1^2\) | 1.022       | 1    | 5.39    | 0.068   |
| \(X_2^2\) | 0.211       | 1    | 3.67    | 0.114   |
| Model ** | –           | 5    | 40.66   | 0.000   |

* df, degree of freedom. ** adjusted \(r^2\) of the model is 0.952.

Analysis of variance (ANOVA) was performed to validate the significance of the model terms (Table 2). Both the linear terms (TAN concentration, \(X_1\); propionate concentration, \(X_2\)) were significant (\(p < 0.001\)). This is in agreement with the model prediction that the lag period increases monotonously according to the increase of both TAN and propionate concentrations (Figure 2). The interaction term (\(X_1X_2\)) was also statistically significant (\(p < 0.05\), while the quadratic terms were only marginally significant (\(p < 0.1\)). The significance of the interaction could be confirmed by the response surface (Figure 2) that the region with higher TAN and propionate concentration (i.e., over 3.5 g TAN/L and 5.0 g propionate/L) showed the steepest slope for the lag period response.
where, R

Table 3) and no significant lack-of-fit was detected (adjusted R

propionate concentration. This model fitted well with the observed data (adjusted R

The second and the third highest methane yield values (0.35 L/g acetate) were obtained in trials 1 and 2, respectively; the top three observations (0.35–0.36 L/g acetate) were made at the lowest TAN concentration. The lowest methane yield (0.14 L/g acetate) was observed in trial 3, which had the highest TAN (5.0 g/L) and propionate (8.0 g/L) concentrations.

After comparing the polynomial models of 1st to 2nd orders, a quadratic model was selected to fit the response of the methane yield:

\[ R_{MY} = 0.350 - 0.0102X_1 + 0.0203X_2 - 0.00481X_1X_2 - 0.00300X_1^2 - 0.00068X_2^2 \]  

(3)

where, \( R_{MY} \) is the estimated response of the methane yield, \( X_1 \) is the TAN concentration, and \( X_2 \) is the propionate concentration. This model fitted well with the observed data (adjusted \( R^2 = 0.846, p = 0.008 \); Table 3) and no significant lack-of-fit was detected (\( p > 0.05 \)). This indicates a good agreement between the experimental and the predicted data (Table 1). The residual plot confirmed that there was no significant pattern with regard to the fitted values (data not shown). Thus, the model (Equation (3)) is suggested to estimate the response surface with accuracy within the study region.

| Term       | Coefficient | df * | F-value | p-Value |
|------------|-------------|------|---------|---------|
| Constant   | 0.350       | –    | –       | –       |
| \( X_1 \)  | –0.0102     | 1    | 50.32   | 0.001   |
| \( X_2 \)  | 0.0203      | 1    | 1.42    | 0.287   |
| \( X_1X_2 \)| –0.00481    | 1    | 2.56    | 0.171   |
| \( X_1^2 \)| –0.00300    | 1    | 0.16    | 0.708   |
| \( X_2^2 \)| –0.00068    | 1    | 0.13    | 0.733   |
| Model **   | –           | 5    | 12.00   | 0.008   |

* df, degree of freedom. ** adjusted \( r^2 \) of the model is 0.846.
ANOVA was performed to validate the significance of the model terms (Table 3). Between the linear terms (TAN concentration, $X_1$; propionate concentration, $X_2$), only the TAN concentration ($X_1$) was significant ($p = 0.001$). This is in agreement with the model prediction that the methane yield decreases primarily due to the increase of the TAN concentration (Figure 3). The interaction ($X_1X_2$) and the quadratic terms ($X_1^2$, $X_2^2$) were not statistically significant ($p > 0.05$) and no proof of interaction was observed from the response surface (Figure 3).

Figure 3. Two-dimensional contour plot of the methane yield within the experimental design.

3.2. Methanogen Populations

The populations of *Archaea* (ARC), *Methanosarcinaceae* (Msc), and *Methanosaetaceae* (Mst) by real-time PCR analysis were shown in Figure 4. *Methanosarcinaceae* and *Methanosaetaceae* are the two known families of methanogens that can directly utilize acetate to produce methane [29], while total archaea covers all methanogenic families of Msc and Mst. Both ARC (>2.4-fold) and Msc (>4.5-fold) showed a distinct growth in all trials, while nearly no growth of Mst was observed during the 60-d period. Between the two acetoclastic methanogen groups (i.e., Msc and Mst), Msc accounted for 74% of the population in the seed inoculum and increased to >92% at the end of the batch incubation (Figure 4b).
would be seriously hampered if acetoclastic methanogens could not overcome the level of inhibition caused by ammonia and/or propionic acid [3].

Methane production is assumed to indicate the growth of methanogens because methanogens produce methane as they grow in number [8]. Depending on the literature, the term methane yield is used to refer to two different parameters: the volume of methane produced per unit substrate provided (L/g substrate provided) or the volume of methane produced per unit substrate consumed (L/g substrate consumed) [8, 34]. In this paper, the term methane yield is used to indicate the former; the latter will be referred to as ‘methane yield per consumption (MYPY)’ later in this paper. The methane yield can be represented as a combination of MYPY and the degree of substrate utilization [34]. The MYPY from the eleven batch trials was estimated as 0.33 ± 0.06 L/g acetate consumed (as chemical oxygen demand (COD) equivalent), similar to that from the control (0.34 L/g) and over 80% to the theoretical maximum (0.40 L/g) [8]. Therefore, the deviation of the methane yield from the RSA experiment was mainly attributable to the different degree of acetate utilization (Table 1). Because sub-optimal environmental conditions may lead to an incomplete consumption of the substrate in batch cultures [35], the presence of the two inhibitors at different concentrations must have been the reason why the residual acetate concentrations varied, and in turn, the methane yield changed.

The two models (Equations (2) and (3)) showed different responses with respect to the TAN and propionate levels. The model for the lag period (Equation (2)) predicted that the lag period imposed to the RSA trials is attributable to the inhibitory effect of ammonia and/or propionate on the acetoclastic consortia (Table 1). The anaerobic process would fail or the efficiency of the process would be seriously hampered if acetoclastic methanogens could not overcome the level of inhibition caused by ammonia and/or propionic acid [3].

4. Discussion

In this study, RSA was conducted to estimate the surface of response variables by testing polynomial models from lower (linear) to higher (quadratic) orders. Two separate response variables were assessed to generate response surface models: the lag period (Figure 2) and the methane yield (Figure 3). The lag period was determined at the intercept where a line tangential to the methane production curve crosses the time axis [30] (Figure 1). The lag period (i.e., the adaptation time) is likely to reflect the time for the methanogens to overcome the inhibition imposed at the beginning stage of the incubation [31]. The lag phase occurs immediately after inoculation and is a period of adaptation of cells to a new environment [32]. This is the time for microorganisms to reorganize their molecular constituents and synthesize new enzymes depending on the composition of nutrients. In this study, there was no difference in environmental conditions between the batch trials and the inoculum system, except for the initial substrate concentration (both systems were fed with 12 g acetate/L) and the inhibitor concentrations (i.e., ammonia and propionate). The substantial lag period of the control (9.4 d; see the footnote of Table 1) could be attributed to substrate inhibition. The initial acetate level of 12 g/L or 200 mM was above the substrate inhibition constants of 4.6 mM (acetate-acclimatized sludge) and 8.3 mM (Methanosarcina barkeri) reported in the literature [33]. On the other hand, the extra lag period imposed to the RSA trials is attributable to the inhibitory effect of ammonia and/or propionate on the acetoclastic consortia (Table 1). The anaerobic process would fail or the efficiency of the process would be seriously hampered if acetoclastic methanogens could not overcome the level of inhibition caused by ammonia and/or propionic acid [3].
increases monotonously according to the increase of both TAN and propionate concentrations (Figure 2). The conditions for the shortest and the longest lag period were clearly represented at the boundary conditions (i.e., 2.0 g TAN/L and 2.0 g propionate/L for the former, 5.0 g TAN/L and 8.0 g propionate/L for the latter). The response surface demonstrated that both inhibitors contributed to the lag period (Figure 2), which was also confirmed by the similar p-value levels for $X_1$ and $X_2$ from the ANOVA (Table 2). The statistical significance ($p < 0.05$) of the interaction term ($X_1X_2$) indicated that the two inhibitors (ammonia and propionate) are likely to interact synergistically (i.e., positive coefficient of this term). Accordingly, the region with higher TAN and propionate concentrations (i.e., over 3.5 g TAN/L and 5.0 g propionate/L) showed the steepest slope for the lag period response (upper right corner of Figure 2a).

The response model for the methane yield (Equation (3)) exhibited a different pattern. The response surface contour implied that the TAN concentration affected the methane yield more clearly than the propionate concentration did (Figure 3), and no distinct interaction (neither positive nor negative) between the two parameters was noticed from the response surface (Figure 3) or the ANOVA (term $X_1X_2$, Table 3). Instead, the methane yield seems to have depended more significantly on the TAN concentration (Figure 3). This means that ammonia imposes a negative effect on the acetate utilization at lower concentrations, probably by affecting the substrate affinity level.

The pH is regarded as a determining factor for certain types of inhibition for anaerobic digestion [3]. As a weak base, ammonia tends to dissociate into ammonium ($\text{NH}_4^+$) in aqueous solution, with more dissociation at a lower pH. Between the two forms, free ammonia ($\text{NH}_3$), the undissociated form, has been ascribed to the toxicity for methanogenesis [1]. Likewise, the undissociated propionic acid ($\text{C}_2\text{H}_5\text{COOH}$), preferably formed at a lower pH, has been suggested as the more toxic form than the dissociated propionate ($\text{C}_2\text{H}_5\text{COO}^-$) [5]. Therefore, the inhibitory effects of the two chemicals to the pH in an opposite direction. In this study, the initial pH of the batch experiments was set to 6.8, where 0.4% of total ammonia and 1.2% of total propionic acid are expected to be at the more toxic, undissociated forms. On the other hand, the pH at the end of the batch trials were 8.1–8.5 (Table 1), where the undissociated forms account for 0.7–1.8% total ammonia and only 0.02%–0.05% total propionic acid. These trends correspond well with our results that propionate inhibition was more significant for the lag period (Table 2), which was determined at earlier stage of the batch culture, than for the methane yield (Table 3), which was governed by the residual acetate concentration at the end of the reaction.

The degree of inhibition detected in this study varied significantly according to the two inhibitor levels. No direct comparison of these results to the literature could be made because this is the first study, to the best of our knowledge, to investigate the co-existing effects of the two inhibitors on acetate-utilizing methanogenesis. Individual effects of ammonia and propionate have been reported: 1.7–14 g TAN/L [1,3,8,20,36] and 0.8–8.0 g propionate/L [5,16,37,38] as inhibition threshold. In this study, more than 50% inhibition of methane yield and maximum methane production rate was detected from trials 3 and 8, where [TAN] = 5.0 g/L and [propionate] = 5.0 or 8.0 g/L (Table 1). Considering that acetate is the most important intermediate in the anaerobic food chain, minimizing the inhibitory effects of these two chemicals would be crucial for an efficient operation of an anaerobic digester. Because ammonia is often generated in the digester when a protein-rich substrate, such as agricultural waste and wastewater, is degraded, a well-functioning digester is likely to have a considerable level of TAN. Therefore, keeping the propionate concentration as low as possible by avoiding overloading and balancing the propionate formation and oxidation would be a practical strategy to minimize the combined inhibition [37,39]. Using the models obtained in this study would provide some quantitative projections of the inhibitions potentially imposed by ammonia and propionate.

In this study, Msc was evidently the major methanogens that converted acetate to methane (Figure 4). The competition for acetate between Msc and Mst was well documented based on their growth kinetics [6] and various case studies [29,40,41]. Msc, an $R$-strategist, has a high maximum growth rate ($R$) but a poor substrate affinity ($K$), while Mst, an $K$-strategist, has a high substrate...
affinity but a lower maximum growth rate [6]. Thus Msc has been found dominant groups in a relatively high residual acetic acid concentration [29,40]. The high initial acetate concentration in the batch system (12 g/L) should have been the key factor that favored the growth of Msc in this study. Comparison between the inhibition trials (trials 1–9) indicated that less growth of Msc was observed with higher propionate concentrations and less growth of Mst was noticed with higher TAN concentrations (Figure 4a). However, these trends were not statistically significant and further investigation is necessary to confirm these trends.

Author Contributions: S.H. was the principal investigator of this project and conceived the experiments. S.G.S. and T.H.D. performed the experiments. S.G.S., J.L. and T.H.D. analyzed the data. S.G.S. and J.L. wrote the manuscript. S.G.S., S.I.K. and S.H. revised the manuscript. All authors read, reviewed, and approved the manuscript.

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