Mathematical models and bacterial communities for ammonia toxicity in mesophilic anaerobes not acclimated to high concentrations of ammonia

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ABSTRACT

In this study, we evaluated ammonia toxicity in mesophilic anaerobic digestion at various pH values and total ammonia nitrogen (TAN) concentrations. We performed anaerobic toxicity assays (ATAs) to evaluate the toxicity effects of TAN and pH on mesophilic anaerobic digestion. Modeling based on the results of the ATAs indicated that the specific methanogenic activity (SMA) decreased by 30% at a TAN concentration higher than 3.0 g/L compared to a TAN concentration of 0 g/L. In addition, the highest SMA for a given TAN level (0.5–10.0 g/L) was observed at a pH of around 7.6. The results of bacterial community analyses showed that the diversity and richness of microorganisms with increasing TAN concentration were decreased. Chloroflexi and Synergistetes were the dominant phyla at TAN concentrations less than 3.0 g/L, and Firmicutes was the dominant phylum at TAN concentrations higher than 3.0 g/L, implying that the ammonia toxicity concentration may influence the kind of dominant species. In conclusion, to start a stable mesophilic anaerobic digestion concerning ammonia toxicity, a TAN concentration less than 3.0 g/L is preferable.

Key words | anaerobic toxicity assay, bacterial community, mesophilic anaerobic digestion, specific methanogenic activity, total ammonia nitrogen

INTRODUCTION

Currently, urban regions and industrial sections generate a huge amount of organic waste (Angelidaki & Ellegaard 2003). Organic waste storage, transportation, and disposal produce many environmental issues, such as odor and greenhouse gas emissions and water and soil contamination. Stringent environmental regulations are prohibiting or discouraging some traditional disposal methods, such as landfills and ocean dumping (Oh et al. 2008), and the treatment or disposal of organic waste is becoming more costly. Therefore, development of environmentally friendly, energy-efficient waste disposal processes is an urgent challenge.

Fortunately, anaerobic digestion can achieve the dual benefits of resource recovery and waste stabilization (Weiland 2005). Anaerobic digestion has several advantages: it requires less energy than alternatives and is highly efficient in using certain types of organic materials. Moreover, anaerobic digestion is relatively simple and straightforward to implement (Kim et al. 2002; Sung & Liu 2003).

However, the organic compounds found in agricultural waste (livestock, poultry, etc.), municipal waste, and food waste have a high nitrogen content (Ward et al. 2014). In addition, the pretreatment of organic compounds releases high concentrations of ammonium ions (NH₄⁺) through the increased degradation of N-containing organic matter (Kim et al. 2003; Park et al. 2014, 2016).

Biogas production and organic removal efficiency can be reduced by high ammonia concentrations during anaerobic digestion (Park & Kim 2016). Total ammonia nitrogen (TAN) concentrations of 1.7–1.8 g/L were completely inhibitory for unacclimated inocula at 35 °C, although the inhibitory TAN level can increase to 3.0 g/L at 35 °C with acclimation (Yenigun & Demirel 2015). Ammonia inhibition has been reported in the range of 1.5–3.0 g/L of TAN above pH 7.4, whereas ammonia (TAN) in excess of 3.0 g/L has been claimed to be toxic irrespective of pH (Calli et al. 2005).

Ammonium (NH₄⁺) and free ammonia (NH₃) are the two principal forms of inorganic ammonia in aqueous solution. Sprott et al. (1984) reported that ammonium inhibits methanogenesis by directly inactivating methane-synthesizing enzymes. On the other hand, free ammonia has been...
suggested as the main cause of this inhibition because it is freely membrane permeable (Kadam & Boone 1996). The hydrophilic ammonia molecule can diffuse passively into cells through cell pores and cause proton imbalance (Cui 2014; Cui et al. 2014). Furthermore, the ammonium/ammonia ratio is pH-dependent. The pH value not only measures hydrogen ion concentrations, but also determines the composition of TAN. Whereas ammonium and hydrogen ions are the major species at low pH values, ammonia and hydroxyl ions become dominant at high pH. Sung & Liu (2005) reported that a TAN concentration of 8–13 g/L caused 100% inhibition at pH 6.5–8.0 in thermophilic anaerobic digestion. However, only a few studies have used mathematical models to evaluate TAN toxicity at mesophilic temperatures and different TAN and pH levels (Hafner & Bisogni 2009).

In addition, a different microbial composition in the wastewater with different ammonia concentration can lead to different metabolic activities. Therefore, it is worthwhile investigating the relationships between bacterial community and treatment system.

In this study, we evaluated the effect of TAN on mesophilic anaerobic digestion with respect to acute ammonia inhibition at different pH and TAN concentrations. Moreover, we further elucidated our experimental results with mathematical models and used pyrosequencing analyses to investigate the composition of the bacterial communities in anaerobic digestion with different ammonia concentrations.

**MATERIAL AND METHODS**

**Batch anaerobic toxicity assay**

We performed anaerobic toxicity assays (ATAs) to study the combined acute toxic effects of TAN and pH on anaerobic microorganisms collected from the mesophilic anaerobic digester in a wastewater treatment plant in Suwon City, Korea. We stored the seed sludge in the laboratory at atmospheric temperature after transfer from the plant and used it for the ATAs.

We injected 25 mL of synthetic wastewater and seed sludge into a 125 mL serum bottle. The initial glucose concentration in each bottle was 1.5 g COD/L for all the tests. We also injected microelements known to have a positive effect on microorganism activity (Kim & Speece 2002). A NaHCO₃ concentration of 6 g/L in the serum bottle was used to prevent pH decrease.

Before sealing the bottle, we removed all internal oxygen with a 60-s nitrogen purge in order to maintain anaerobic digestion. We then sealed the bottles with butyl rubber stoppers and incubated them in a shaker chamber set at 200 rpm and 35°C. At the beginning of each experiment, we adjusted the pH and TAN concentrations to the desired values, and we conducted each experiment in duplicate. We also prepared a control of the seed sludge without synthetic wastewater in order to estimate the biogas produced from only the substrate. We performed ATAs at four different pH values of 6.7, 7.6, 8.5, and 9.2 and TAN concentrations of 0.5, 1, 3, 6, and 10.0 g/L, using NH₄HCO₃ for each condition.

We used a manometer to measure gas emissions by calculating the difference between the inner pressure of the bottle and that of the atmosphere. During the early part of the reaction (2 days), we measured emissions every 6 h. After that, we measured emissions at 2 and 4 d, stopping when we had confirmed that gas production had stopped. The methane production was calculated as shown in the equation (Equation (1)) (Park & Kim 2015).

\[
V_{CH4} = C_1(V_1 + V_0) - C_0V_0.
\]

where, \(V_{CH4}\) is the volume of methane produced (mL), \(C_1\) is the methane content (%) at sampling time, \(C_0\) is the methane content (%) at the previous sampling time, \(V_1\) is the biogas volume measured by a syringe (mL) and \(V_0\) is the gas phase volume of the reactor (mL).

**Analytical procedures**

We used standard methods to determine the amounts of ammonium ions, chemical oxygen demand (COD), total nitrogen, total phosphate, alkalinity, total suspended solid, and volatile suspended solids (VSS) (APHA 2005). We measured pH using a pH meter (PHM92 Laboratory, Radiometer Analytical, Bagsnaerd, Denmark) and FA according to the formula of Anthonisen et al. (1976). The methane content was determined using gas chromatography (STAR 3400 CX, Varian, USA) with a capillary column (DB-5, 0.53-mm diameter, 30-m length, J&W Scientific) at 29°C (Park et al. 2014) and a flame ionization detector. The injector and detector temperatures were 150°C and 200°C, respectively. The initial oven temperature was maintained at 30°C for 2 min and then increased by 10°C/min to 50°C. The carrier gas was nitrogen with a flow rate of 20 mL/min. The injection volume of methane gas in the GC was 10μL.
Calculations for the Gompertz model were done with programs in MATLAB (MATLAB R2008a, MathWorks, USA).

Model description

Gompertz model

The Gompertz model can be used to describe bacterial growth (Zwietering et al. 1990). Based on the study of Sung & Liu (2003), which related bacterial growth to metabolic biogas production, we used this equation to describe cumulative methane production in the ATAs according to the following equation (Sung & Liu 2003):

\[ M = P \cdot \exp \left[ - \exp \left( \frac{B \cdot R' \cdot e}{P} (\gamma - t) + 1 \right) \right] \]  (2)

where, \( M \) (mL) is the cumulative methane production at incubation time \( t \) (day), \( \gamma \) (day) is the lag-phase time, \( P \) (mL) is the methane production potential, \( B \) (g VSS) is the total biomass in the bottle, and \( R' \) (mL CH₄/g VSS day) is the specific methanogenic activity (SMA).

For each pH–TAN combination in the batch toxicity assay, we recorded the corrected \( M \) versus \( t \). Given \( B \), we estimated the model parameters in Equation (2) using least-square estimation. \( R' \) represents the SMA of a batch (mL CH₄/g VSS day), the slope of the cumulative methane production versus time, which we further used as an inhibition response for modeling the inhibition effects of TAN and pH.

Extended Monod equation

At a specific pH, the effect of ammonium or ammonia on methanogens is determined using the extended Monod equation (Sung & Liu 2003):

\[ R' = R_m \left( 1 - \frac{I}{I^*} \right)^n \left( \frac{S}{S + K_s(1 - (I/I^*)^m)} \right) \]  (3)

where, \( R' \) (mL CH₄/g VSS day as in Equation (2)) is the SMA determined from batch tests at an inhibitor concentration of \( I \) (g/L as ammonia), \( R_m \) (mL CH₄/g VSS day) is the maximum SMA (without inhibitor) at a specific pH, \( S \) (g/L as COD) is the substrate concentration, \( K_s \) (g/L as COD) is the half-saturation constant, \( I^* \) (g/L as ammonia) is the lethal ammonium and ammonia concentration beyond which the reaction cannot proceed, and \( n \) and \( m \) are coefficients.

The extended Monod equation includes two parameters (\( n \) and \( m \)) to embody the inhibition effects, and they are generally used to determine the type of inhibition. This equation can represent six common patterns of inhibition (Han & Levenspiel 1987): (1) noncompetitive inhibition, where \( n > 0 \) and \( m = 0 \); non-competitive inhibition is a type of enzyme inhibition where the inhibitor reduces the activity of the enzyme and binds equally well to the enzyme whether or not it has already bound the substrate; (2) competitive inhibition, where \( n = 0 \) and \( m < 0 \); competitive inhibition is a form of enzyme inhibition where binding of the inhibitor to the active site on the enzyme prevents binding of the substrate and vice versa; (3) generalized uncompetitive inhibition, where \( n > m > 0 \); (4) uncompetitive inhibition, where \( n = m > 0 \); (5) competitive inhibition known as anti-competitive inhibition, taking place when an enzyme inhibitor binds only to the complex formed between the enzyme and the substrate (the E-S complex); (6) general inhibition, for any \( n > 0 \) and \( m < 0 \). Theoretically, \( m \) influences the inhibition pattern when the inhibitor is at low concentration, and \( n \) determines the inhibition pattern at high concentrations.

Michaelis pH function

We analyzed the effect of pH on SMA at a given TAN concentration using the Michaelis pH function (Angelidaki et al. 1995; Sung & Liu 2003), which provides a central value of \( R_0 \) (when pH = 0.5 \( \cdot (pH_L + pH_H) \)), as shown in Equation (4). The parameters \( pK_L \) and \( pK_H \) determine the location and shape of the pH inhibition curves, respectively.

\[ R' = R_0 \frac{1 + 2 \times 10^{0.5(pH-pK_H)}}{1 + 10^{(pH-pK_L)} + 10^{(pK_H-pK_L)}} \]  (4)

where, \( R' \) is the SMA of a batch (mL CH₄/g VSS day), \( R_0 \) is the SMA at optimum pH at a specified TAN level (mL CH₄/g VSS day), and \( pK_L \) and \( pK_H \) are the lower and higher pH drop-offs, respectively.

Microbial community

In this study, we collected four sludge samples from serum bottles at the end of the SMA. We placed the sludge samples in an ice box following field collection and stored them at \(-60^\circ\)C until pyrosequencing analysis. We performed polymerase chain reaction (PCR) amplification using primers targeting the V1 to V3 regions of the 16S rRNA gene with extracted DNA under the following conditions: initial
denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and final elongation at 72 °C for 5 min. We confirmed the PCR product using 2% agarose gel electrophoresis and visualized it under the Gel Doc system (BioRad, Hercules, CA, USA). The amplified products were purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). We assessed the quality and product size on a Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA) using a DNA 7500 chip.

We sorted the obtained reads from the different samples based on the unique barcodes of each PCR product. We removed the sequences of the barcode, linker, and primers from the original sequencing reads and discarded any reads with two or more ambiguous nucleotides, a low quality score (average score <25), or a read shorter than 300 bp. We detected potential chimera sequences using the Bellero phon method, which compares the BLASTN search results between the forward half and reverse half sequences (Huber et al. 2004). After removing chimeric sequences, we assigned the taxonomic classification of each read using the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net).

RESULTS AND DISCUSSION

Effect of TAN on anaerobic microorganisms under the influence of pH

Figure 1 shows the results of the SMA at different TAN concentrations and pH levels with anaerobic microorganisms.

![Figure 1](image)

Figure 1 | SMA of anaerobic microorganisms at different TAN concentrations and pH levels.

We used the extended Monod equation to model ammonia inhibition at a given pH and the non-linear least-square regression function in MATLAB (MATLAB R2008a, MathWorks, USA) to perform least-square estimation of the parameters \( I^* \), \( R_{m,n} \), \( n \), and \( m \) using Equation (3). The solid lines in the figure are the response to the prediction of Equation (3) with the fitted parameters \( I^* \), \( R_{m,n} \), \( n \), and \( m \). The measured values are a little lower than the model-predicted values at a pH 8.5. Nevertheless, other model-predicted values showed a good agreement with measurements. The highest SMA was determined at pH 7.6. At a lower pH of 6.7 and a higher pH of 8.5 the SMA was much lower. Thus the optimum pH for methane production could be around 7.6. However, the SMA value decreased with increasing TAN concentration independent of pH. In particular, the SMA value decreased rapidly at TAN concentrations higher than 3.0 g/L. Therefore, a TAN concentration higher than 3.0 g/L can inhibit growth of anaerobic microorganisms.

Table 1 summarizes the kinetic parameters of \( R_{m,n} \), \( n \), and \( m \) for the extended Monod equation for anaerobic microorganisms at various pH conditions. Although the value of \( n \) at different pH values did not change significantly for the different TAN concentrations, it did decrease when the pH was lower or higher than 7.6. On the other hand, the value of parameter \( m \) increased when the pH was lower or higher than 7.6; although, its range was much larger than that of \( n \). The values of \( n \) and \( m \) were fixed for determining inhibition type, which indicates the uncompetitive inhibition of methane formation if the values of \( n \) and \( m \) are higher than 0. In this study, the values of \( n \) and \( m \) were higher than 0. Therefore, inhibition by high TAN concentration in anaerobic digestion was uncompetitive between substrate and inhibitor (ammonia). SMA and \( I^* \) showed the highest values at pH 7.6. Like the \( n \) change, SMA and \( I^* \) decreased when the pH was lower or higher than 7.6. In terms of the extended Monod equation used in this study, all parameter values except parameter \( m \) decreased with an increase in inhibitor concentration. The results are similar to those of Sung & Liu (2003), who reported that the parameters \( I^* \), \( R_{m,n} \) and \( n \) but not \( m \) in the extended Monod equation decrease with a TAN concentration higher than 3.0 g/L and a pH above 9.0 or below 6.0 in thermophilic anaerobic digestion (Sung & Liu 2003).

Effect of pH on TAN inhibition

To investigate the role of pH in ammonia inhibition, we modeled SMAs vs. pH values at given TAN concentrations...
using the normalized Michaelis pH function (Equation (4)). We used the non-linear least-square function in MATLAB (MATLAB R2008a, MathWorks, USA) to perform non-linear regression. Figure 2 shows the effect of pH on SMA at various concentrations of TAN with a mesophilic methanogenic consortium. The SMA decreased at TAN higher than 3.0 g/L. In addition, SMA values at a pH lower than 7.0 or higher than 8.5 decreased 30–50% compared to the levels at pH 7.6 because both TAN and pH inhibited the anaerobic microorganisms. The highest SMA for any given TAN level was observed at a pH of around 7.6.

Table 2 summarizes the kinetic parameters of $pK_L$, $pK_H$, and $R_0$ in the normalized Michaelis pH function at various conditions for anaerobic microorganisms. With the $R^2$ values shown in Table 2, the Michaelis model well predicted the pH effect, which could be considered another inhibition factor independent of TAN. As a result, the optimum pH range and $R_0$ values rapidly decreased at any TAN concentration higher than 3.0 g/L due to decreased anaerobic microorganism activity.

**Table 2 | Summary of the kinetic parameters $pK_L$, $pK_H$, and $R_0$ from the normalized Michaelis pH function at various conditions for anaerobic microorganisms**

<table>
<thead>
<tr>
<th>TAN (g/L)</th>
<th>$pK_L$</th>
<th>$pK_H$</th>
<th>$R_0$ (mL CH$_4$/g VSS day)</th>
<th>SSE*</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>6.510</td>
<td>8.305</td>
<td>2.5640</td>
<td>1.E-01</td>
<td>0.9155</td>
</tr>
<tr>
<td>1</td>
<td>6.645</td>
<td>8.291</td>
<td>2.3542</td>
<td>1.E-01</td>
<td>0.9056</td>
</tr>
<tr>
<td>3</td>
<td>6.729</td>
<td>7.789</td>
<td>2.1574</td>
<td>1.E-01</td>
<td>0.9225</td>
</tr>
<tr>
<td>6</td>
<td>7.058</td>
<td>7.697</td>
<td>1.3541</td>
<td>8.E-02</td>
<td>0.9462</td>
</tr>
<tr>
<td>10</td>
<td>7.139</td>
<td>7.653</td>
<td>0.7952</td>
<td>3.E-02</td>
<td>0.9356</td>
</tr>
</tbody>
</table>

*SSE is the sum of squared error.

**Effects of TAN and pH on SMA**

To study the combined effects of TAN and pH on SMA, we carried out a quadratic regression of ammonia inhibition for methanogens using TAN and pH as predictors. The ranges of pH (5.0–10.0) and TAN concentration (0–15 g/L) chosen for the experiment illustrated in Figure 3 were much wider than those observed in the experiments. We predicted the peripheral area using the quadratic model. As is evident from the contours, higher SMA was observed...
at lower TAN concentrations and around neutral pH for anaerobic microorganisms, suggesting that both pH and TAN inhibit methanogens. TAN concentrations less than 1.5 g/L did not show any detrimental effect on SMA, whereas TAN concentrations higher than 3.0 g/L caused appreciable inhibition. Therefore, to start a stable mesophilic anaerobic digestion of high-ammonia wastewater, it is necessary to maintain a lower than 3.0 g/L ammonia concentration and pH 7.5–7.7.

### Variation in microbiology

#### Richness and diversity at different ammonia concentrations

Table 3 presents our statistical calculations based on pyrosequencing analysis. We identified a total of 16,343 reads from the collected sludge. The average mean read length was 380 bp which had been also reported in other studies (Hur et al. 2013; Kim et al. 2014). We estimated that the value was reliable in this study. The largest number of operational taxonomic units (OTUs) was determined at a TAN concentration of 1.0 g/L, and the smallest number of OTUs was determined at a TAN concentration of 0.5 g/L. OTUs are the most commonly used microbial diversity unit. The richness estimations of ACE and Chao 1 indicate that a TAN concentration of 1.0 g/L promotes the highest richness and a TAN concentration of 10.0 g/L results in the lowest richness among all samples. ACE and Chao 1 are representing the richness. The OTUs and richness (ACE and Chao 1) of TAN concentration of 1.0 g/L were the highest. After that richness was decreasing as TAN concentration increased, demonstrating that the microorganisms were negatively impacted by a high TAN concentration. Based on the rarefaction curves (which is a technique to assess species richness) and OTUs, the diversities of anaerobic microorganisms from high to low were 1.0 > 3.0 > 10.0 > 0.5 (g/L) (Figure 4 and Table 3). The predominant phyla differed with TAN concentration. The results indicate that different TAN concentrations produce significant differences in richness and diversity.

### Investigation of bacterial community

Figure 5 shows the composition of the predominant bacterial community according to phyla. The predominant phylum differed according to TAN concentration. The most predominant phylum at TAN concentrations of 0.5 (30% of total microorganisms number) and 1.0 (29% of total microorganisms number) g/L was Chloroflexus, whereas the most predominant phylum at TAN concentrations of 3.0 (24% of total microorganisms number) and 10.0 (29% of total microorganisms number) g/L was Firmicutes. Chloroflexi and Synergistetes were the dominant communities at a TAN concentration of lower than 3.0 g/L, and Firmicutes was the dominant community at a TAN concentration higher than 3.0 g/L.

Synergistetes are obligate anaerobic microorganisms and methane can be formed by syntrophic associations between Synergistetes and methanogens (Jumas-Bilak et al. 2009; Fernandes et al. 2014). The prevalence of Synergistetes at ammonia concentrations of 0.5 and 1.0 g/L was 24% and

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**Table 3 | Description of pyrosequencing analysis**

<table>
<thead>
<tr>
<th>Ammonia concentration (g/L)</th>
<th>Valid reads</th>
<th>Mean read length (bp)</th>
<th>Observed OTUs</th>
<th>ACE estimation</th>
<th>Chao 1 estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>4,063</td>
<td>383.0</td>
<td>623</td>
<td>1,649</td>
<td>1,167</td>
</tr>
<tr>
<td>1.0</td>
<td>4,178</td>
<td>381.7</td>
<td>684</td>
<td>1,735</td>
<td>1,241</td>
</tr>
<tr>
<td>3.0</td>
<td>4,834</td>
<td>378.7</td>
<td>673</td>
<td>1,564</td>
<td>1,209</td>
</tr>
<tr>
<td>10.0</td>
<td>3,268</td>
<td>376.4</td>
<td>643</td>
<td>1,501</td>
<td>1,105</td>
</tr>
</tbody>
</table>
22%, respectively, but it decreased to 13% at 3.0 g/L and 11% at 10.0 g/L. Apparently, *Synergistetes* die out at high TAN concentrations. Many *Firmicutes* produce endospores, which are resistant to desiccation and can survive extreme conditions. *Firmicutes* included various aerobic, anaerobic, and facultative aerobic microorganisms and are found in various environments, and include some notable pathogens (*Haakensen et al.* 2008). Therefore, it was determined that *Firmicutes* could be dominant at TAN higher than 3.0 g/L. Gas production decreases with increasing TAN because of the rapid decreases in *Chloroflexi* and *Synergistetes*.

### CONCLUSIONS

This study evaluated the acute toxicity of ammonia and the influence of pH in mesophilic anaerobic digestion. Our conclusions are as follows.

1. The analysis of the ATA data using the extended Monod model revealed characteristics of uncompetitive inhibition for methane production from complex substrates.

2. The SMA results at various TAN concentrations showed higher SMA at lower TAN concentrations (<1.5 g/L); higher TAN concentrations (>3.0 g/L) caused obvious inhibition of methane formation by anaerobic microorganisms.

3. The results of the bacterial community analysis showed that *Chloroflexi* and *Synergistetes* were the dominant phyla at TAN concentrations lower than 3.0 g/L and *Firmicutes* was the dominant phylum at TAN concentrations higher than 3.0 g/L.

4. Finally, to start a stable mesophilic anaerobic digestion, with respect to ammonia toxicity a TAN concentration less than 3.0 g/L is preferable at a pH around neutral.

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