# Ammonia and pH Inhibition in Anaerobic Treatment

**N. Eldem[1], I. Ozturk[1], E. Soyer[1], B. Calli[2], B. Mertoglu[2], and O. Akgiray[2]**
[1] Istanbul Technical University, Faculty of Civil Engineering, Department of Environmental Engineering, Maslak 80626, Istanbul, Turkey
[2] Marmara University, Faculty of Engineering, Department of Environmental Engineering, Goztepe 34722, Istanbul, Turkey

**Abstract** Ammonia is a metabolic product in the decomposition of wastes containing proteins, and is known to have an inhibitory effect on methanogenesis. This paper reports the results of our studies on the inhibitory effects of ammonia/ammonium and pH on methanogenic activity under mesophilic conditions. Two independent sets of experiments were carried out using seeds from two different full-scale industrial anaerobic treatment reactors. One of the seeds ("Sludge A") was obtained from an upflow anaerobic sludge-blanket (UASB) reactor treating the waste from a potato chips production plant. The second sludge seed ("Sludge B") was from an expanded granular sludge-blanket (EGSB) reactor treating a corn processing waste. Experiments were carried out at four different pH values (6.8, 7.4, 7.8, 8.4) and with six different TAN (Total Ammonia Nitrogen) values (262 mg/L (control), 1000, 1500, 2000, 2500, 3000). A new model was developed and found to give a very good fit to the data. To investigate the variations in microbial diversity in the sludge seeds, FISH (Fluorescent In-Situ Hybridization) experiments were carried out.
**Keywords** Ammonia inhibition; anaerobic treatment; FISH; methanogenesis; pH inhibition; toxicity.

## Introduction

Ammonia is a product generated during the anaerobic treatment of wastewaters containing proteins. While ammonia is an essential nutrient for methanogens, high levels of ammonia can be toxic to methanogenic archaea. According to McCarty and McKinney (1961) and McCarty (1964), TAN (Total Ammonia Nitrogen) concentrations between 50 and 200 mg/L are beneficial to anaerobic processes, whereas concentrations between 1500 and 3000 mg/L are inhibitory at pH levels above 7.4, and concentrations higher than 3000 mg/L are toxic at all pH values. On the other hand, other investigators reported satisfactory anaerobic treatment at concentrations well above 1500 mg/L and in combination with pH values above 7.5 (Van Velsen, 1979; De Baere et al., 1984; Koster, 1986; Sprott and Patel, 1986; Koster and Lettinga, 1988).

Ammonia exists is two forms in solution, i.e. as unionized (free) ammonia and ammonium ion. It is reported that free ammonia is a more powerful inhibitor than ammonium ion (Koster and Koomen, 1988; Angelidaki and Ahring, 1993). As a result, the inhibition observed at a given TAN concentration depends on pH. In addition to this "indirect" effect of pH on methanogenic activity, pH may also have a direct inhibitory effect. Included in the "direct" pH effect is the inhibition caused by the unionized forms of the volatile fatty acids. High pH values, on the other hand, may also reduce methane production rate, as methanogens may not be able to utilize the ionized forms of the volatile fatty acids as substrate. Any possible direct interaction of hydroxide or hydrogen ions with parts of organisms' methanogenic apparatus exposed to the medium would also be included in the direct pH effect. According to the terminology adopted here, in the absence of inhibitory levels of ammonia and ammonium, the observed variation of methanogenic activity with pH would be due only to the direct pH effect.

## Experimental

The influences of pH and ammonia on methane production in the anaerobic treatment of an acetic-propionic-butyric acid mixture in batch reactors operated at 37 $^{o}$C (mesophilic conditions) were investigated. Two independent sets of experiments were carried out using seeds from two different full-scale industrial anaerobic treatment reactors. One of the seeds was obtained from an upflow anaerobic sludge-blanket (UASB) reactor treating the waste from a potato chips production plant. This sludge seed will be called "Sludge A" in what follows. The second sludge seed was from an expanded granular sludge-blanket (EGSB) reactor treating a corn processing waste. This sludge will be referred to as "Sludge B." Sludge B had a more granular structure compared to Sludge A. Neither sludge was specially acclimated to ammonia in the lab before the inhibition tests.

Anaerobic inhibition tests were carried out at 37±1 $^{o}$C in 100 ml serum vials. The biogas produced by the biomass was measured by using a water displacement technique. Three separate vials (triple runs) were used for each pH/TAN combination. Four different pH values (6.8, 7.4, 7.8, 8.4) and six different TAN values (262 mg/L (control), 1000, 1500, 2000, 2500, 3000) were used in the study (see Fig.1). In each set of experiments (for each sludge), therefore, a total number of 72 vials were used for the 24 different pH/TAN combinations. The pH values in the reactors were controlled using 1N HCl or 8N NaOH solutions. TAN concentrations were adjusted by adding $NH_4Cl$. The experiments were continued until complete cessation of methane production. Once an experiment was ended, the final conditions (pH, TAN, etc.) in the corresponding vials were measured within the same day. Further details about the experimental procedure and apparatus can be found in Eldem *et al*. (2004).

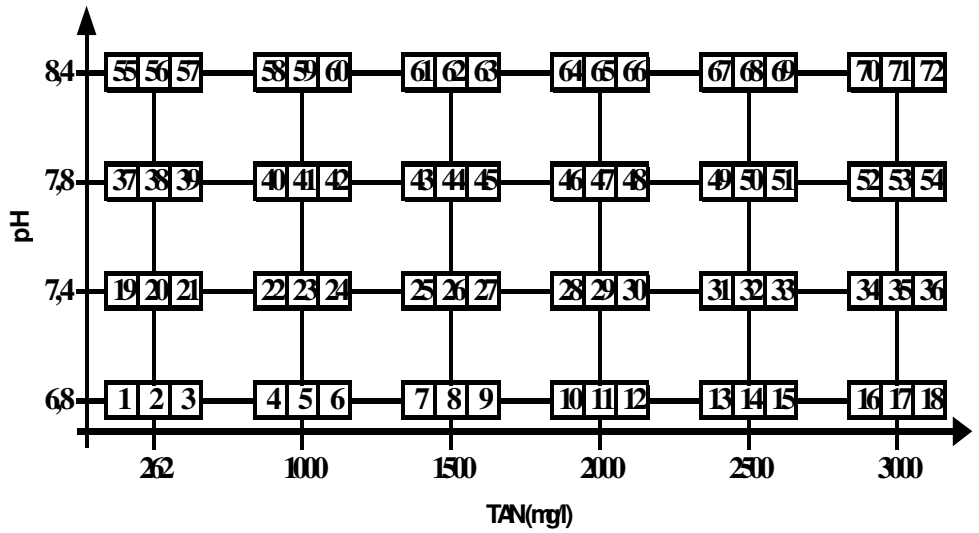



**Figure 1** The experimental matrix and vial numbers (three vials for each pH/TAN combination).

To investigate the variations in microbial diversity in the sludge seeds, FISH (Fluorescent In-situ Hybridization) experiments were carried out. The sludge samples were fixed overnight in 4% paraformaldehyde-phosphate-buffered saline at 4$^{o}$C. Fixed cells were spotted on gelatin-coated [0.1% gelatin and 0.01% $KCr(SO_4)_2$] multiwell glass slides (10 wells/slide; 4 µl of sample/well) and allowed to dry in a sterile hood.

Hybridizations were performed at 46°C for 2 h with a hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, pH 8.0, 0.01% SDS) containing each labeled probe (30 ng/well for Cy3 and TRITC 50 ng/well for FLUOS) (MWG Biotech, Ebersberg). Formamide was added to ensure the optimal hybridization stringency (for details, see Calli *et al*., 2003). After hybridization, unbound oligonucleotides were removed by rinsing with washing buffer containing the same components of the hybridization buffer except the probes. For detection of all DNA, 4,6-diamidino-2-phenylindole

(DAPI) was added to the wash buffer (100 µl of 0.1% DAPI). Slides were subsequently incubated at 48 °C for 10 min with washing buffer, rinsed briefly with $ddH_2O$ and immediately air-dried. Vectashield (Vector Laboratories) was used to prevent photo bleaching. The slides were examined with an Olympus BX50 microscope. Digital images of the slides were taken with a digital camera.

## Results and Discussion

A number of investigators (e.g. Lay *et al*., 1998; Liu and Sung, 2002; Sung and Liu, 2003) employed the modified Gompertz equation as a model for cumulative methane production:

$$M = P \cdot \exp\left\{-\exp\left[\frac{R \cdot e}{P}(\lambda - t) + 1\right]\right\} \quad (1)$$

where M = cumulative methane production, mL/(gr VS); e = exp(1); R = methane production rate, mL/(gr VS)(day); P = methane production potential, mL/(gr VS); λ = lag-phase time, days. The applicability of this model to the data obtained in this work was investigated. Nonlinear regression calculations were carried out to find the values of P, R, and λ for each pH/TAN combination. It was concluded that this equation fits a large fraction of the data very well. An example is provided in Fig.2. At certain combinations of high pH and high TAN values, however, the cumulative methane production data manifested an initial "hump" (e.g. Fig.3) that cannot be predicted using the Gompertz equation. With Sludge A, this happened at pH ≥ 7.8 and with TAN ≥ 1000-1500 mg/L. With Sludge B, this phenomenon was observed only at pH = 8.4 and with TAN ≥ 1000 mg/L. It was found that the application of the Gompertz equation in such cases yields model parameters that are not physically meaningful (e.g. negative λ values; see dashed curve in Fig.3). In such cases, therefore, the model parameters were determined by ignoring the hump and using only the data for larger times. The application of this modified approach is illustrated by the solid curve in Fig.3. The R values obtained in this manner are in good agreement with the maximum slopes observed during the experiments. Whenever an initial hump was present (as in Fig.3), the maximum rate was observed always after the re-start of methane formation (i.e. not during the initial period of activity).

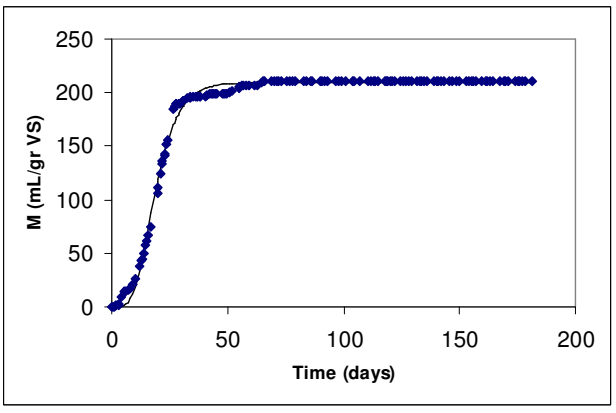



**Figure 2** Data and model line for pH=8.4, TAN = 262 mg/L (control), Sludge A.

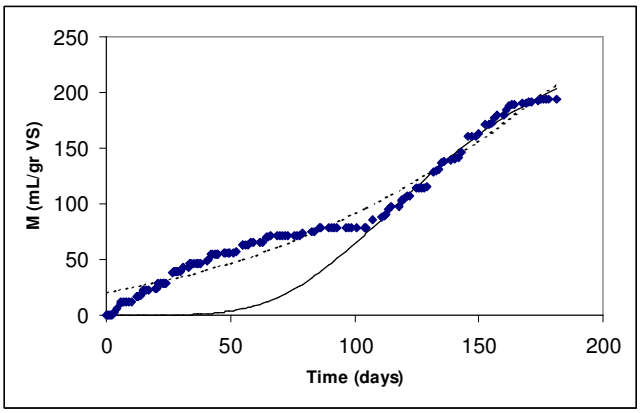



**Figure 3** Data for pH=8.4, TAN = 1000 mg/L, Sludge A. Dashed curve is obtained by using all the data; solid curve by ignoring the hump.

The normalized Michaelis pH function was used to account for the direct inhibitory effect of pH, i.e. for the variation of rate in the control vials:

$$R\,(\,in\ the\ controls) = \frac{K_m'\left(1 + 2\times 10^{0.5(\,pK_1 - pK_2\,)}\right)}{1 + 10^{(\,pH - pK_2\,)} + 10^{(\,pK_1 - pH\,)}} \quad (2)$$

where $K'_m$ is referred to as "the maximum specific methane production rate." The parameters $pK_1$ and $pK_2$ are referred to as the lower pH drop-off value and the higher pH drop-off value, respectively. Drop-off is defined as the pH at which methanogenic activity is reduced by 50%.

Next, Eq.2 was fitted to the R values for the control vials obtaining the following results. Sludge A: $pK_1 = 6.37$, $pK_2 = 8.58$, and $K'_m = 16.59$ ($r^2 = 0.9914$). Sludge B: $pK_1 = 5.30$, $pK_2 = 8.36$, and $K'_m = 112.5$ ($r^2 = 0.9987$).

Using the maximum rate (R) values for all pH/TAN combinations, the parameters a, b, and $R_m$ in the following equation were then determined by nonlinear regression:

$$R = R_m \exp[-(aF + b(1-F))TAN]\frac{\left(1+2\times10^{0.5(pK_1-pK_2)}\right)}{1+10^{(pH-pK_2)}+10^{(pK_1-pH)}} \quad (3)$$

where $R_m$ denotes the maximum methane production rate, i.e. the rate that would be observed in the absence of the inhibitor and at pH = $(pK_1+pK_2)/2$. The parameters a, b, $R_m$, $pK_1$, and $pK_2$ are true constants, independent of pH and ammonia/ammonium concentrations. The fraction F of unionized (free) ammonia nitrogen is calculated by

$$F = \left(1+10^{(pK_a-pH)}\right)^{-1} \quad (4)$$

where $K_a$ = ionization constant of the ammonium ion. It should be noted that F(TAN) and (1-F)TAN represent free ammonia and the ammonium ion concentrations, respectively. The relative magnitudes of a and b, therefore, represent the relative toxicity of ammonia and ammonium.

Using Eq.3, Eq.4, and the previously determined values of $pK_1$ and $pK_2$, the following parameters values were obtained by means of nonlinear regression: Sludge A: $R_m = 19.318$, a = 0.00756, b = 0.000191 ($r^2 = 0.9209$). Sludge B: $R_m = 123.13$, a = 0.00407, b = 0.000154 ($r^2 = 0.9841$). Model predictions using these parameter values are shown in Figures 4 and 5.

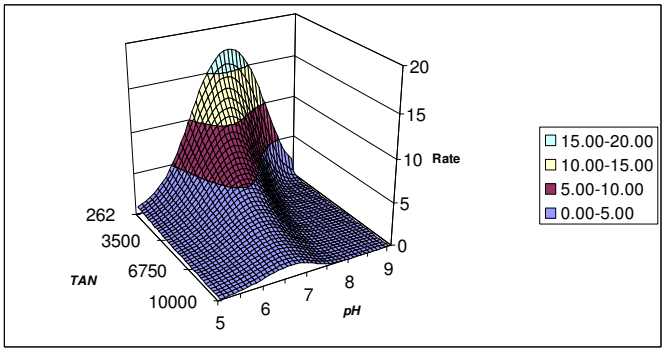



**Figure 4** Methane production rate as predicted by Equation 3 for Sludge A.

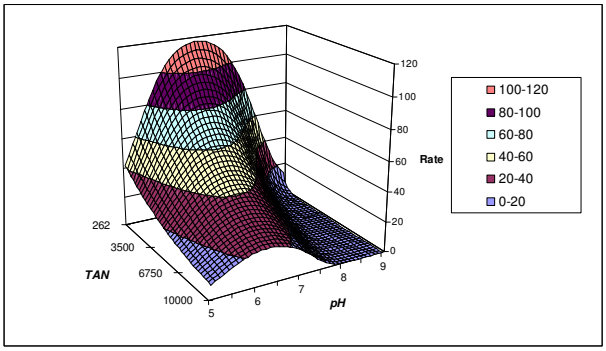



**Figure 5** Methane production rate as predicted by Equation 3 for Sludge B.

An inspection of the horizontal axes shows that the same amount of substrate was converted to methane in a considerably shorter time with Sludge B, i.e. methane production rate of Sludge B was higher than that of Sludge A. From the model developed in this work (Eq.3), the maximum uninhibited rate for Sludge A and Sludge B were 19.3 and 123.1 (mL CH4)/(gr VSS)(day), respectively. The higher methanogenic activity of Sludge B is consistent with fluorescent in-situ hybridization (FISH) observations that Sludge B contained a higher

concentration of methanogens than Sludge A. It should also be emphasized that Sludge B had a more granular structure than Sludge A. The present experiments revealed that Sludge B not only manifested higher methane production rates, but it was also more resistant to ammonia inhibition: Lag-phase times were shorter (zero for Sludge B, up to two days with Sludge A), and the presence of the initial hump was observed at higher pH values with Sludge B.

In-situ hybridization results have indicated that archaeal cells representing the methanogens were intensively dominant in both sludge seeds. It is revealed that Methanosaeta was the major acetoclastic methanogenic archaea present in both seeds (Figures 6-7). Some methanogenic populations of Methanosarcina were also detected in Sludge B while hydrogentrophic methanogens were virtually absent.

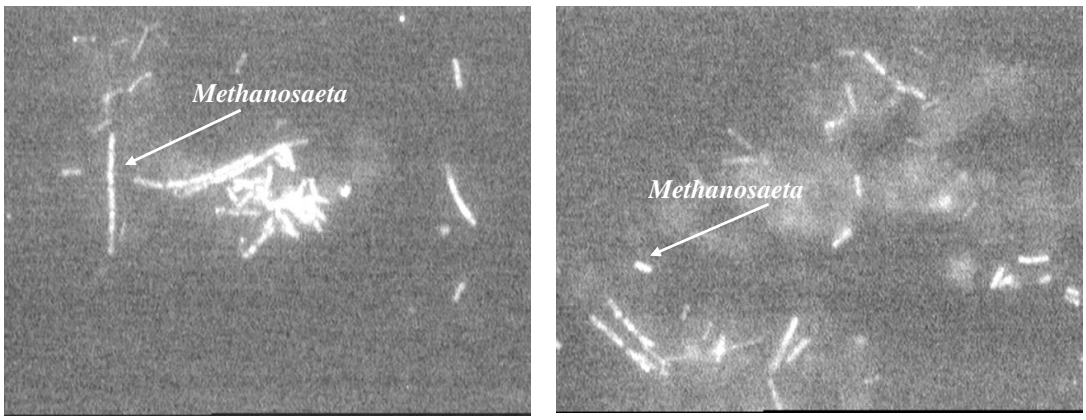



**Figure 6** Microscopic pictures of Sludge A after the FISH experiment.

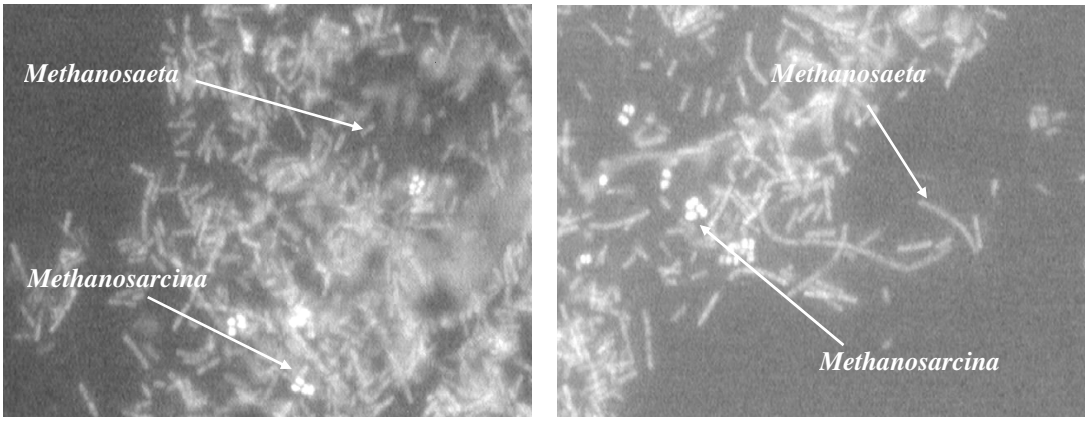



**Figure 7** Microscopic pictures of Sludge B after the FISH experiment.

## Conclusions

Batch experiments were carried out with two different sludge seeds to study the inhibitory effects of ammonia and pH on methanogenic activity. With both sludges, the maximum methane formation rate decreased consistently as TAN was increased from 262 mg/L up to 3000 mg/L at constant pH. Similarly, with both sludges, the methane production potential decreased consistently as TAN was increased at constant pH. This effect was less pronounced for Sludge B which had a more granular structure. With both sludges, free ammonia was found to be much more inhibitory than the

ammonium ion. While it is clear that free ammonia is (more) inhibitory to methanogenesis, it is not possible to state conclusively whether the ammonium ion has a direct inhibitory effect or the inhibitory effect ascribed to the ion (the non-zero value of the parameter b) is an artificial consequence of the nonlinear regression calculations. Although neither sludge was exposed to high levels of ammonia before the batch tests, no significant initial lag-phase was observed in these experiments. With Sludge A, methane formation always started within at most two days. An initial lag-phase was completely absent with (the more granular) Sludge B. With both sludges, at certain high pH and high TAN values, methanogenic activity ceased within about 1 to 3 months; and then activity restarted and methane formation rate reached its maximum values after this period. If the experiments had not been continued following this initial period, this reactivation phenomenon would not have been observed. While the modified Gompertz equation gave a good fit to the data when an initial hump was absent, this modelling approach is not directly applicable when an initial hump is present. A simple novel model was used to fit the maximum rate data. The model has the following advantages: The model parameters are true constants (independent of pH and TAN). Secondly, the data for all pH and TAN combinations are represented by a single equation.

## References


Angelidaki, I. and Ahring, B.K. (1993). Thermophilic anaerobic digestion of livestock waste: Effect of ammonia. *Applied Microbiology and Biotechnology*, 38(4), 560-564.

Calli B., Mertoglu B., Tas N., Inanc B., Yenigun O. and Ozturk I. (2003). Investigation of variations in microbial diversity in anaerobic reactors treating landfill leachate. *Water Science Technology*, 48(4), pp.105-112.

De Baere, L.A., Devocht, M., Van Assche, P. and Verstraete, V. (1984). Influence of high NaCl and NH4Cl salt levels on methanogenic associations. *Water Research*, 18(5), 543-548.

Eldem, N., Oztürk, I., Soyer, E., Calli, B, and Akgiray, O. (2004). Ammonia and pH inhibition in anaerobic treatment of wastewaters, Part I: Experimental. *J. Environ. Sci. Health, Part A*. Vol.A39, No.9 (in press).

Eldem, N., Akgiray, O., Ozturk, I., Soyer, E., and Calli, B (2004). Ammonia and pH inhibition in anaerobic treatment of wastewaters, Part II: Model Development. *J. Environ. Sci. Health, Part A*. Vol.A39, No.9 (in press).

Koster, I.W.; Lettinga, G. (1984). The influence of ammonium-nitrogen on the specific activity of palletized methanogenic sludge. *Agric. Wastes*, 9(3), 205-216.

Koster, I.W. (1986). Characteristics of the pH-influenced adaptation of methanogenic sludge to ammonia toxicity. *Journal of Chemical Technology and Biotechnology*, 36, 445-455.

Koster, I.W. and Koomen, E. (1988). Ammonia inhibition of the maximum growth rate of hydrogenotrophic methanogens at various pH-levels and temperatures. *Applied Microbiology and Biotechnology*, 28(4-5), 500-505. Chapter 5.

Koster, I.W.; Lettinga, G. (1988). Anaerobic digestion at extreme ammonia concentrations. *Biological Wastes*, 25(1), 51-59.

Lay, J. J., Li, Y. Y., and Noike, T. (1998). *Water Env. Research*, Vol:70, No:5, pp.1075-1082.

Liu, T. and Sung, S. (2002). *Water Science and Technology*, Vol:45, No:10, pp.113-120.

McCarty, P.L. (1964). Anaerobic waste treatment fundamentals, Part III. Toxic materials and their control. *Public Works* 95(12), 91-94.

McCarty, P.L. and McKinney, R.E. (1961). Salt toxicity in anaerobic treatment. *Journal of Water Pollution Control Federation* 1961, 33(4), 399-415.

Sprott, G.D. and Patel, G.B. (1986). Ammonia toxicity in pure cultures of methanogenic bacteria, *Systematic and Applied Microbiology*, 7, 358-363.

Sung, S. and Liu, T. (2003). *Chemosphere*, Vol:53, pp.43-52.

Van Velsen, A.F.M. (1979). Adaptation of metanogenic sludge to high ammonia-nitrogen concentrations. *Water Research*, 13(10), 995-999.