**The impact of ammonia nitrogen concentration and zeolite addition on the specific
methanogenic activity of granular and flocculent anaerobic sludges**

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ABSTRACT

This work presents the effect of ammonia nitrogen concentration and zeolite addition on the specific methanogenic activity (SMA) of different anaerobic sludges with various physical structures (granular and flocculent), operating in batch conditions. Piggery, malting production and urban sludges derived from full-scale anaerobic reactors were tested in the experiment as the source of inoculum in batch digesters. It was found that piggery sludge was the most affected by the increase of ammonia nitrogen concentration while malting producing and municipal sludges were less affected. In general, the addition of zeolite at doses in the range of 0.01-0.1 g /g VSS reduced the inhibitory effect of N-NH_4^+ for piggery sludge (P.S.). For this sludge, the propionic:acetic ratio increased when the concentration of N-NH_4^+ increased, indicating that methanogenesis was affected. Finally, a study of the microbial population involved in this study for P.S. by using 16S rRNA based molecular techniques revealed a presence of microorganisms following the order: *Methanococcaceae* > *Methanosarcina* > *Methanosaeta*.

Keywords: Zeolite, ammonia nitrogen, specific methanogenic activity, piggery sludge, malting sludge, urban sludge, flocculent, granular

INTRODUCTION

Ammonia is a nutrient for the growth of bacteria involved in the anaerobic digestion process, but at certain concentrations it acts as an inhibitor.^[1] Zeeman et al.^[2] found that thermophilic anaerobic digestion of cow manure may be inhibited at ammonia nitrogen concentration >3

g/L. The suggested cause of the inhibition of methanogenesis when the medium pH increased was ammonia.^[3,4] According to Wiegant and Zeeman^[5] ammonia acts as an inhibitor of methane formation from CO₂ and H₂, having a lower effect on methane formation from acetate but leads the inhibition of propionate break-down acting as an inhibitor of the acetate-consuming methanogens. Sterling et al.^[6] found a decrease of hydrogen and methane production with an increase in the ammonia concentration, causing the total inhibition at 3 g/L. Salerno et al.^[7] also demonstrated the inhibitory effect on hydrogen production at a concentration of 2 g N/L. In the anaerobic digestion of a mixture of cattle manure and proteins at concentrations in the range of 50-300 mM, Robbins et al.^[8] concluded that acetate to methane pass-way was the most affected. In the anaerobic degradation of swine manure using continuously stirred digesters at ammonia concentration of 6 g-N/L and at a hydraulic retention time of 15 days and temperatures of 37°C, 45°C, 55°C and 60°C, Hansen et al.^[9] obtained methane yields of 188, 141, 67 and 22 mL CH₄/g VS, respectively, showing the process inhibition. Koster and Lettinga^[10] studied the stability of adapted granular sludge in a UASB reactor applied to potato juice. Methanogenesis occurred at ammonia nitrogen concentration of 11.8 g/L but ceased at 16 g/L. The influence of ammonia on the activity and attachment of methanogenic associations was also studied.^[11] Two reactors received shock loadings while the other two were adapted to increasing ammonia concentrations. The first set of reactors were inhibited at a concentration of 30 g/L while in the second set 50 % of inhibition occurred at 45 g/L. Omil et al.^[12] and Vidal et al.^[13] found similar inhibitory concentrations. Sossa et al.^[14] studied the effect of ammonia on the specific methanogenic activity (SMA) in a biofilm enriched by methylaminotrophic methane producing *Archaea*. Doses of 48.8, 73.8, 98.8, 148.8, 248.8, 448.8 and 848.8 mg of ammonia nitrogen/L were added to flasks containing ceramic rings colonized for a 30-day-old experimental biofilm. Inhibition appeared at 148.8 mg/L. They concluded that the inhibitory effect of ammonia acted in two ways by affecting directly the methane synthesis enzyme or by diffusion into the

cell causing proton imbalance and potassium deficiency.^[14] Calli et al.^[15] demonstrated that propionate degrading acetogenic bacteria are more sensitive than *Archaea* to ammonia. Calli et al.^[16] also compared the ammonia inhibition of UASB, upflow filters and hybrid reactors treating landfill leachates and concluded that anaerobic filters and hybrid reactors were more efficient.

Different alternatives have been researched to overcome the inhibition caused by ammonia. Jaffer et al.^[17] and Demirer et al.^[18] proposed the addition of magnesium salts to the digesters to obtain struvite (magnesium-ammonium phosphate) by precipitation. Gangagni-Rao et al.^[19] introduced a stripper with a UASB reactor treating poultry litter leachate to diminish the inhibitory effect of ammonia. A COD removal of 96% and a methane yield of 0.26 L/g COD removed were achieved at an organic loading rate of 18.5 g COD/L day and a hydraulic retention time of 12 h, while in the control the maximum organic loading accepted without inhibition was 13.6 g/L d at a hydraulic retention time of 16 h. Borja et al.^[20] compared the anaerobic digestion of cow manure using natural zeolite as support with a control in batch digesters. The kinetic constant of the control decreased considerably with the increase in ammonia concentration, while in the reactor, which contained zeolite, the kinetic constant remained practically invariable and the ammonia concentration decreased due to the ionic exchange effect of zeolite. In continuous fluidized bed digesters with cattle manure as substrate the reactor with zeolite as support was more efficient than a control with suspended biomass^[21]. In thermophilic anaerobic digestion of cattle manure, zeolite addition was capable of reducing the inhibitory effect of free ammonia.^[22] Cintoli et al.^[23] used natural zeolite for the pre-treatment of piggery wastewater and ammonia concentration decreased from 1500 to 300 mg/L reducing the toxicity towards anaerobic microbial population and improving the performance of a UASB reactor treating this waste. Milan et al.^[24] studied the application of zeolite doses in the range of 0.2-10 g/L in batch anaerobic digestion of piggery

waste, obtaining the best results at doses of 2-4 g/L. Tada et al.^[25] compared different types of zeolites and other materials at ammonia nitrogen concentrations as high as 4500 mg/L and the best results were obtained with the use of mordenite. Montalvo et al.^[26] studied different doses and procedures of zeolite dosage in batch and in continuous mixed anaerobic digesters applied to synthetic and piggery waste. They found that the addition of zeolite at the influent at a dose of 1 g/L was the most effective procedure. Kotsopoulus et al.^[27] studied the addition of zeolite to thermophilic anaerobic digesters working with piggery waste at doses of 0, 4, 8 and 12 g/L. They found that at doses of between 8 and 12 g-Zeolite/L, both methane production and volatile solids removal were significantly higher in comparison to the control, the optimum dose being 4 g-Zeolite/L.

On the other hand, even though methanogenic population dynamics in anaerobic treatment systems have been looked at in more recent studies by using molecular methods,^[14,16] our understanding is still limited under stressful conditions as elevated concentrations of inhibitory compounds. Ammonia nitrogen is one of the most common toxic substances encountered during anaerobic treatment of several wastes.

The subject of the present work was to evaluate the effect of ammonia nitrogen concentration on the specific methanogenic activity (SMA) and the influence of zeolite addition to ameliorate the inhibition caused by ammonia by using three different sludge types as inoculum. In this study, microbial communities were also monitored to gradually increasing N-NH_4^+ levels using 16S rRNA based molecular techniques for some selected conditions.

MATERIALS AND METHODS

Inoculum characteristics

Three different inoculums were selected for the study: Piggery Sludge (P.S.), Malting Sludge (M.S.) and Urban Sludge (U.S). The inoculums were collected from full-scale anaerobic digestion plants in operation. The characteristics of the inoculums used are shown in Table 1.

Zeolite characteristics

The natural zeolite used with a particle size of about 0.5 – 1.0 mm was obtained from Tasajera deposits, in the Province of Villaclara, Cuba. The characteristics, chemical and phase composition of the zeolite used are given in Table 2.

Experimental procedure

The experiments were carried out at a temperature of 35°C and at pH in the range of 6.8-7.2. The synthetic substrate used was methanol at a concentration of 80 g/L and a basal medium. The basal medium was composed of: Na₂S (0.2 mL of a solution of 4 g/L); NaHCO₃ (0.5 mL of a solution of 10 g/L); and L-cysteine (0.1 mL of a solution of 50 g/l). The inoculums were adapted to the substrate during a period of one month. After the adaptation of the inoculums to the substrate, ammonia chloride (NH₄Cl) and zeolite were added at doses of: 0.1, 0.2, 1.0, 3.0, 6.0 and 12.0 g/L of reactor and 0.01, 0.1 and 1.0 g/g VSS, respectively.

Equipment

The experiments were carried out in serum bottles of 50 mL effective volume. Each serum bottle was inoculated with 10 mL of inoculum. During the experiment the contents of the

bottles were mixed by magnetic stirrers at 120 rpm. The bottles were hermetically closed and the methane gas produced was measured by the displacement of a 3 M NaOH solution in order to remove the CO₂ and measure only the methane gas produced to determine the values of SMA.

Analytical methods

The specific methanogenic activity (SMA) was determined by the method suggested by Milan et al.^[28] Chemical Oxygen Demand (COD), ammonia nitrogen, total volatile fatty acids (TVFA), total and volatile suspended solids (TSS and VSS respectively) and pH were determined according to Standard Methods.^[29] Volatile fatty acids (VFA) composition of some selected samples was determined by gas chromatography, according to the procedure described in detail elsewhere.^[26,28]

16sS rRNA analysis

The developed microbial communities for some selected conditions in the experiments carried out with P.S. as inoculum were analysed by biological molecular techniques using a quantitative Dot Bolt Hybridization. The samples were treated prior to rRNA extraction. Target groups were identified with oligonucleotide probes by using EUB338: bacteria, SRB385: sulphate reducing bacteria (SRB), ARCH915: *Archaea*, MC1109: *Methanococcaceae*, MS821: *Methanosarcina*, MX825: *Methanosaeta*.^[30]

RESULTS AND DISCUSSION

Effect of zeolite on the SMA values

Figure 1 shows the variation of the SMA values with the zeolite doses. The highest SMA values were found for U.S. in comparison with the rest of the inoculums. On the other hand, the minimum SMA values were observed for P.S., due to the higher concentration of non-methanogenic microorganisms (71.55%) and a lower concentration of methanogenic microorganisms (28.45%) present in this inoculum. The addition of zeolite at doses of 0.01-0.1 g/g VSS to the P.S. and U.S. (flocculent structures) increased the SMA values, probably due to the immobilization effect of the microorganisms on the zeolite surface that enhanced the metabolic activity.^{20,24,26,31,32} It is recognized and widely reported that microorganisms have a tendency to adhere to solid surfaces enhancing their activity.^{11,16,33} A previous study²⁷ revealed that zeolite addition at doses of 8-12 g/L of waste considerably increased the methane production in thermophilic (55°C) anaerobic digestion of piggery waste. These results appear to be caused by the addition of zeolite, which adsorbs ammonia, thus having an effect not only on the toxicity of ammonia and on the C/N proportion but also on the regulation of acidity (pH) of the waste studied. In the case of M.S. (granulated sludge) the addition of zeolite at doses in the range of 0.01 and 0.1 did not increase the SMA values and, even at higher doses (1 g/g VSS) a decrease in the SMA value was observed. At 1 g/g VSS, the SMA decreased in the three cases studied, probably due to an increase in the concentration of OH⁻ on the surface of the biofilm.^[16]

Effect of ammonia on the SMA values

Figure 2 shows the effect of the ammonia nitrogen on the SMA for P.S., M.S. and U.S. inoculums. The SMA for P.S. decreased 75% of its initial value when the N-NH₄⁺ concentration increased from 0 to 1 g/L. However, in the case of M.S. the decrease was only

14.7% and for U.S. a decrease in the SMA was not observed. At a concentration of N-NH_4^+ of 3 g/L, the specific methanogenic activity of piggery sludge (P.S.) was not measurable, while in the case of M.S., the SMA decreased 18.7% and for U.S. the SMA only decreased 0.8%. When the concentration of N-NH_4^+ increased to 6 g/L, the SMA of M.S. inoculum decreased 23.6% with respect to its initial value while for U.S. the SMA only decreased 3%. At a concentration of 9 g N-NH_4^+ the value of SMA decreased 68.3% and 25.7% for M.S. and U.S. inoculums respectively and, finally, at 12 g N-NH_4^+ the SMA of M.S. and U.S. decreased 81.3% and 68.3%, respectively. These SMA values were significantly higher than those obtained for P.S. without the addition of N-NH_4^+ . These results demonstrated that the resistance of anaerobic microorganisms to high N-NH_4^+ concentrations does not depend on the sludge structure but on the microbial composition and the adaptability and acclimatization of the microorganisms to high ammonia nitrogen concentrations.^[9,14,34,35]

Effect of zeolite addition at different N-NH_4^+ concentrations on the SMA values

Figures 3, 4 and 5 show the effect of ammonia nitrogen concentration and zeolite addition on the SMA for P.S., M.S. and U.S., respectively. In the case of P.S., the addition of zeolite increased the SMA significantly with respect to the control when the concentration of N-NH_4^+ increased. It can be observed in Figure 3 that the differences in SMA values due to the inhibitory effect of N-NH_4^+ decreased when the zeolite concentration increased. Therefore, the addition of zeolite reduced the inhibitory effect of N-NH_4^+ . This was probably due to the fact that zeolite acted as an ion exchanger reducing the concentrations of free ammonium and in turn the concentration of ammonia nitrogen.^[21,22,25,36,37] Similar behaviour was observed in the anaerobic digestion process of synthetic wastewater using digested piggery sludge as inoculum. This process was also favoured by the addition of zeolite at doses of between 0.05 and 0.30 g/g of VSS, the optimum value being 0.10 g/g VSS.^[31]

In the case of M.S. (Figure 4), zeolite caused a reduction of the inhibitory effect of N-NH_4^+ at doses of 0.01 g/g VSS and N-NH_4^+ concentrations of 0.1 and 0.2 g/L. When the concentration of ammonia nitrogen increased to 6 g/L, the addition of 0.1 g Zeolite/g VSS overcame the inhibition of methanogenesis and as a consequence the value of SMA was practically the same as when the N-NH_4^+ was not added. At a zeolite concentration of 1 g/g VSS there were no differences in the SMA values for 6 and 9 g/L of N-NH_4^+ , probably because this dosage of zeolite was capable of reducing the inhibition. At a N-NH_4^+ concentration of 9 g/L, the increase of zeolite doses increase the resistance to the inhibition. At a N-NH_4^+ concentration of 6 g/L, there was an increase in the inhibition resistance up to a dose of 0.1 g/g VSS; however, a further increase in the dose of zeolite determined a decrease of the inhibition resistance, probably because an excess of the zeolite doses caused damage to the microorganism cells as was previously discussed. As was reported beforehand,^[31] the increase of the zeolite doses per gram of inoculum (VSS) used may have affected the mass transfer of organic matter, either nutrients and metabolites, in the vicinity of zeolite particles and the microorganisms associated.

In the case of U.S. (Figure 5), zeolite addition improved the resistance of microorganisms to the inhibition caused by N-NH_4^+ . At a concentration of N-NH_4^+ of 6 g/L, the addition of zeolite up to 1 g/g VSS favoured the resistance of microorganisms to the inhibition and the values of SMA were higher compared to the controls. However, when the concentration of ammonia nitrogen increased to 9 g/L, inhibition occurred and the values of SMA were slightly lower compared to the control. Therefore, zeolite performs three roles when it is added in an anaerobic digestion process: it is a support for microorganism immobilization, determining a higher efficiency for nutrient uptake and metabolite outlet; it is an ionic exchanger preventing the toxic effect of ammonium and it can act as an inhibitor of the bacterial activity at a given concentration.^[20,24,25,27,32,33,36,38]

Evaluation of the effects of $N\text{-NH}_4^+$ and zeolite on the behaviour of P.S. sludge

Based on the experimental results previously obtained a more detailed study was carried out with P.S. inoculum to determine the origin of its lower resistance to the inhibitory effect of $N\text{-NH}_4^+$. Figure 6 shows the variation of total volatile fatty acid composition and concentration with the $N\text{-NH}_4^+$ concentration and the zeolite addition for the experiments carried out with P.S. Maximum values of acetic acid were found at the highest doses of zeolite and for the lowest $N\text{-NH}_4^+$ concentration (0.2 g/L). In the case of propionic acid, its concentration was higher when ammonia nitrogen concentrations were low and when zeolite addition was the highest although the $N\text{-NH}_4^+$ concentration was also higher. Therefore, it appears that when inhibition started the concentration of acetic and propionic acids decreased. However, it was also found that the propionic:acetic ratio increased when the concentration of $N\text{-NH}_4^+$ increased, indicating that the methanogenesis was affected. Butyric acid concentration increased when the concentration of $N\text{-NH}_4^+$ increased, but also when the zeolite doses increased to a value on which the toxic effect of zeolite appeared. Therefore, the presence of butyric acid may be linked to inhibition due to the presence of ammonia nitrogen and an excess of zeolite addition.^[16,24,34]

Effect of $N\text{-NH}_4^+$ concentration and zeolite addition on the microbial community for P.S. sludge

In order to study the effect of $N\text{-NH}_4^+$ concentration and zeolite addition on the microbial population for P.S. sludge, which showed the worst behaviour against ammonia inhibition, dot blot hybridization of extracted rRNA of some representative conditions was carried out. As can be observed in Table 3, the P.S. anaerobic sludge as inoculum presented a high

percentage of bacteria and it changed for all conditions tested, observing a higher percentage of *Archaea* in all cases. The condition that showed the highest percentage of *Archaea* corresponded to digesters without Nitrogen addition and 1.0 g of zeolite/g VSS, followed by the same zeolite dose plus 1.0 g of Nitrogen/L. In general, the *Archaea* percentage was not greatly affected by the addition of nitrogen.

Table 4 shows the composition of the microbial population for the different conditions analysed. There was a stable composition of microorganisms in the percentage. A slight decrease only for 2.0 g/L of Nitrogen was observed. As far as analyzed population is concerned, *Methanosaeta* was the least present, while *Methanococcacea* was the most present for all conditions studied, with a slightly higher effect of natural zeolite dose, up to 1.0 g/L of Nitrogen addition. *Methanosarcina* was also present in high percentages, but always lower than *Methanococcacea*. In general, the order in which they were present was: *Methanococcacea* > *Methanosarcina* > *Methanosaeta*. The presence of SRB was only favoured in control digesters and digesters with 0.2 g/L of Nitrogen and zeolite dose of 1.0 g/g VSS.

CONCLUSIONS

From the experimental results obtained, it may be concluded that the highest methanogenic activity was found in U.S., while the lowest one was found in P.S. At the same time, the highest resistance to the inhibition by N-NH_4^+ was found in U.S. while the lowest was observed for P.S. Zeolite contributed to overcoming the inhibition by ammonia nitrogen at doses in the range of 0.01-0.1 g/g VSS. However, at a concentration of 1 g/g VSS, a toxic effect by zeolite addition appeared.

In general, the percentage of Archaea was always higher than the percentage of bacteria for all conditions tested for P.S. sludge. A study of the microbial population of P.S. carried out by dot-blot hybridization of extracted rRNA revealed the percentage of major microorganisms according to the following order: *Methanococcacea* > *Methanosarcina* > *Methanosaeta*. The presence of SRB was only favoured in control digesters and digesters with 0.2 g/L of N-NH₄⁺ and zeolite dose of 1.0 g/g VSS.

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Table 1. Characteristics of the piggery (P.S.), malting (M.S.) and urban or municipal (U.S.) anaerobic sludges.

<i>Parameters*</i>	<i>Unit</i>	<i>P.S.</i>	<i>M.S.</i>	<i>U.S.</i>
Volatile solids	mg/g TS	70.5	83.6	59.2
N (Kjeldahl)	mg/g TS	58.0	63.0	49.0
PO ₄ ³⁻	mg/g TS	1.8	1.0	2.1
SMA (to methanol)	g COD-CH ₄ /gVSS d	0.24	1.23	1.32
Structure	-	Flocculent	Granular	Flocculent

* Average values of ten analyses carried out on a lot of sludge

Table 2. Chemical and phase composition of the natural zeolite from Tasajera, Villaclara, Cuba used in the experiments.

Chemical composition (%)		Phase composition (%)	
SiO ₂	58.05	Clinoptilolite	35.0
Al ₂ O ₃	11.94	Mordenite	15.0
Fe ₂ O ₃	4.36	Montmorillonite	30.0
MgO	0.77	Others*	20.0
CaO	5.94		
Na ₂ O	1.50		
K ₂ O	1.20		

* Others – Calcite, Feldespate and Quartz.

Table 3. Percentage of bacteria and *Archaea* of some analyzed conditions selected for P.S. sludge.*

Operating conditions	Initial	N0+Z0	N0+Z1.0	N0.2+Z1.0	N1.0+Z1.0	N2.0+Z1.0	N2.0+Z0
Bacteria (%)	71.55	30.41	8.59	20.29	13.32	30.48	20.85
Archaea (%)	28.45	69.59	91.41	79.71	86.68	69.52	79.15

* Initial: inoculum; N0+Z0: control digesters (without Nitrogen and Zeolite); N0+Z1.0: digesters without Nitrogen and 1.0 g zeolite/g VSS; N0.2+Z1.0: 0.2 g of N/L and zeolite dose of 1.0 g/g VSS; N1.0+Z1.0: 1.0 g of N/L and zeolite dose of 1.0 g/g VSS; N2.0+Z1.0: 2.0 g of N/L and zeolite dose of 1.0 g/g VSS; N2.0+Z0: 2 g of N/L and without added zeolite.

Table 4. Percentage composition (16S RNA) of hybridized microorganisms for some analysed conditions for P.S. sludge.

Microrganisms*	Initial	N0+Z0 (controls)	N0+Z1.0	N0.2+Z1.0	N1.0+Z1.0	N2.0+Z1.0	N2.0+Z0.0
<i>SRB</i>	5.6	17.8	6.7	18.9	12.2	8.9	12.1
<i>Mc</i>	16.6	37.8	54.4	44.4	50.0	44.3	44.5
<i>Ms</i>	7.7	17.7	33.3	37.7	26.7	28.9	28.8
<i>Mx</i>	6.7	17.6	5.5	No detected	12.2	No detected	7.7

* *SRB*: sulphate reducing bacteria; *Mc*: *Methanococcaceae*; *Ms*: *Methanosarcina*;

Mx: *Methanosaeta*. The rest of abbreviations are identical to those shown in Table 3.

Figure captions

Figure 1. Effect of the zeolite doses on the SMA for P.S., M.S. and U.S. sludges.

Figure 2. Effect of N-NH_4^+ concentration on the SMA for P.S., M.S. and U.S.

Figure 3. Effect of zeolite doses at N-NH_4^+ concentrations of 0, 0.1 and 0.2 g/L for P.S

Figure 4. Effect of zeolite doses at N-NH_4^+ concentrations of 0, 0.1, 0.2, 6.0 and 9.0 g/L for M.S.

Figure 5. Effect of zeolite doses at N-NH_4^+ concentrations of 0, 6.0 and 9.0 g/L for U.S.

Figure 6. Effect of zeolite doses and N-NH_4^+ concentration on the volatile acids concentration in some anaerobic tests using P.S. as inoculum.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6