

Article

The Multifunctional Effect of Porous Additives on the Alleviation of Ammonia and Sulfate Co-Inhibition in Anaerobic Digestion

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Abstract: Ammonia and sulfide derived from the reduction of sulfate by the sulfate-reducing bacteria (SRB) are two of the most common inhibitors in anaerobic digestion. Zeolites and bentonites are characterized as porous materials able to adsorb both ammonia and sulfur compounds and seem to be promising candidates as additives in anaerobic digestion to counteract this co-inhibition. In this study, bentonite and zeolite 13X were subjected to alkali modification at different concentrations of NaOH to alter their physicochemical properties, and their effect on the alleviation of ammonia and sulfate co-inhibition in anaerobic digestion of cow manure was examined. The methane production in 13X treatments (13X without NaOH, 13X02-NaOH 0.2 M and 13X1-NaOH 1 M) was elevated by increasing the NaOH concentration in the modification step, resulting in a significance increase by 8.96%, 11.0% and 15.56% in 13X treatments compared to the treatment without additive. Bentonite treatments did not show the same behavior on the toxicity mitigation. The results appear to be influenced by the combined effect of 13X zeolites on the sulfur compounds adsorption and on the increase in pH and Na⁺ concentration in the batch reactors.

Keywords: alleviation, ammonia and sulfate inhibition; anaerobic digestion; zeolite; bentonite



Citation: Tzenos, C.A.; Kalamaras, S.D.; Economou, E.-A.; Romanos, G.E.; Veziri, C.M.; Mitsopoulos, A.; Menexes, G.C.; Sfetsas, T.; Kotsopoulos, T.A. The Multifunctional Effect of Porous Additives on the Alleviation of Ammonia and Sulfate Co-Inhibition in Anaerobic Digestion. *Sustainability* **2023**, *15*, 9994. <https://doi.org/10.3390/su15139994>

Academic Editors: Ranjana Chowdhury and Sudipta De

Received: 15 May 2023
Revised: 12 June 2023
Accepted: 21 June 2023
Published: 24 June 2023



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1. Introduction

Organic wastes are byproducts of many domestic, agricultural and industrial activities and require proper treatment before they are discharged in the environment. Commercially, anaerobic digestion (AD) is a popular method of waste management, as it utilizes various organic wastes to produce biogas, which in turn can be used as a renewable energy source to generate heat and electricity [1]. The treatment of substrates rich in organic content in the absence of oxygen, for the production of biogas, is referred to as AD. Generally, biogas consists mainly of carbon dioxide and methane mixed with trace amounts of other compounds such as ammonia and hydrogen sulfide [2].

The drastic increase in electricity and heating costs, along with the effort to prevent climate change, has led to the development of the international Sustainable Development Goals (SDGs) [3]. Biogas production contributes directly to 12 out of the 17 SDGs. The sustainable management of agricultural, municipal and industrial waste, among others, increases renewable energy production, promotes environmental protection, assists the waste disposal process, and creates employment opportunities [4].

The proper function of a biogas plant and the stable production of biogas are affected by several factors that can disrupt them. Among them, ammonia and sulfide derived from

the reduction of sulfate by the sulfate-reducing bacteria (SRB) have been considered as two of the most important inhibitory factors of AD [5], as ammonia and sulfate are commonly present in many of the wastes.

Sulfate-reducing bacteria (SRB) produce sulfide (H_2S) by consuming sulfate (SO_4^{2-}) alongside with other common organic substrates necessary for the metabolism of methanogenic archaea (MA). Effectively, methanogenesis is inhibited competitively by sulfidogenesis, which can lead to the suppression of methane production. In particular, the growth of SRB is favored over the growth of methanogens due to the thermodynamic and substrate affinity conditions of anaerobic digesters, outcompeting the latter for their common substrates such as H_2 and acetate [6]. The produced H_2S can also be toxic to MA at fairly low concentrations (50 mg L^{-1}) [5]. The production of H_2S is a common problem in biogas plants, so various conventional technologies exist to control it, including metal sulfide precipitation, chemical oxidation, pH regulation, micro-aeration, adsorption and suppression of sulfidogenesis [7,8].

Ammonia is necessary for the growth of all the microorganisms involved in AD, as it is an essential element for synthesis of amino acids, proteins and nucleic acids, but it can also act as an inhibitor at higher concentrations [9]. The anaerobic digestion of substrates rich in compounds containing nitrogen, such as urea and proteins, produces ammonia nitrogen, which in aquatic solutions exists mainly in two forms, as ammonium ion (NH_4^+) and as free ammonia (FA). Of the two forms, FA is widely known to inhibit MA [10]. The suggested mechanism of FA inhibition relies on the passive diffusion of the ammonia molecules into the cells, which can cause proton imbalances and/or potassium deficiencies [11]. The inhibitory effects of FA are well documented in both lab- and full-scale anaerobic digesters, with highly variable critical concentrations depending on the type of digester, the operating temperature (mesophilic, thermophilic) and the available substrates [10]. Many methods have been proposed to counteract ammonia toxicity, including lowering the digester temperature to reduce FA levels, the addition of ammonium-binding inorganic compounds, increasing the C/N ratio, dilution with water and ammonia stripping [9].

Among the different methods for the alleviation of ammonia toxicity, the use of inorganic additives is among the most well documented and effective in lab-scale experiments [6]. Two of the most commonly used inorganic additives are zeolites and bentonites, whose physical and chemical properties have been proven to be beneficial to anaerobic digestion. Zeolites and bentonites are porous aluminosilicates of alkali and alkaline earth cations, their crystalline matrix formed by SiO_4^{4-} and AlO_4^{5-} tetrahedra being linked by shared oxygen atoms. Their most prominent characteristics are the ability to exchange cations (ion-exchange property) and the adsorption of molecules with specific cross-sectional diameter (adsorption property) [12], which can be improved alongside with other characteristics by chemical modification [13]. The effect of bentonite and zeolite on the alleviation of ammonia toxicity has been extensively reported. In an anaerobic digester, with the Na^+ , Ca^{2+} and Mg^{2+} cations present in their crystalline matrix, both act as ion exchangers removing NH_4^+ , thus lowering the concentration of ammonia. Their effect in overcoming the ammonia inhibition of AD is not based solely on their ion-exchange capacity, but also on other properties such as their negative surface charge that allows for effective adsorption of cations along with their porosity and BET surface that can help immobilize and support the microorganisms in AD [12]. Although aluminosilicates have been used to adsorb sulfide and sulfate in aqueous solutions and wastewater [14,15], their effect on the reduction of sulfide inhibition in AD is limited. On the one hand, sulfide and ammonia co-inhibition is commonly present in AD [16], and aluminosilicates seem to be promising candidates, as they are able to adsorb both ammonia and sulfur compounds to counteract this co-inhibition. Additionally, modification of aluminosilicates can increase their adsorption capacity. However, the effectiveness of modified aluminosilicates on the elimination of sulfide and ammonia co-inhibition has not been studied to date.

In the present study, for first time, the multifunctional effect of alkali-modified inorganic additives on the anaerobic process under high ammonia and sulfate concentra-

tions was examined. Particularly, two types of clay minerals, bentonite and zeolite 13X, were treated with various concentrations of sodium hydroxide (NaOH) in order to create additives with different adsorption and ion-exchange properties, and their effect on the enhancement of methane production in the presence of both NH_3 and SO_4^{2-} was evaluated. Additionally, their effect on methane production under only high ammonia concentration (without the presence of high concentration of SO_4^{2-}) was also studied, as this is the most common use of the aluminosilicates, as aforementioned.

2. Materials and Methods

2.1. Inorganic Additives

2.1.1. Bentonite and Zeolite 13X

Two common inorganic additives were chosen for this study, bentonite and zeolite 13X. Bentonite is an adsorbent swelling clay that consists mostly of montmorillonite and in smaller quantities of quartz, volcanic glass, organic matter, gypsum and silver. Bentonite is a sheet-like clay substance that is used in industry to make viscous suspensions and can be utilized as binders, plasticizers, or suspending agents depending on the bentonite/water ratio [17].

Zeolite 13X is a product of the reaction of kaolinite with sodium hydroxide. In particular, kaolinite and sodium hydroxide are combined to create a solid combination that is later heated to melt the components. The final product, also known as zeolite 13X, is obtained after additional processing [18]. The chemical composition of the unmodified zeolite 13X (Molecular Sieves, 13X powder, Thermo Scientific, Waltham, MA, USA) and bentonite (Bentonite Clay for laboratory use, Chem-Lab, Zedelgem, Belgium) was measured with EDS microanalysis and is presented in Table 1.

Table 1. Chemical composition * of unmodified Zeolite 13X and Bentonite.

Constituents	Adsorbents	
	Bentonite	Zeolite 13X
O	47.15	46.3
Si	28.15	22.28
Al	11.45	18.44
Fe	3.325	N.D. **
Cu	2.025	N.D. **
Na	2.425	12.9
Mg	2.275	N.D. **
K	0.775	0.08
Ca	2.425	N.D. **

* All the values are in percentage by weight. ** Not Detected.

2.1.2. Methodology of Alkali Modification of Bentonite and Zeolite 13X

Bentonite (Be without NaOH treatment) and zeolite 13X (13X without NaOH treatment) were subjected to alkali modification with different concentrations of sodium hydroxide (NaOH) solutions to alter their physicochemical properties. The following process was used to prepare alkali-treated zeolites. The zeolite 13X modification resulted in the following additives, 13X02-NaOH 0.2 M treatment and 13X1-NaOH 1 M treatment and the bentonite modification, in Be1-NaOH 1 M treatment. An Erlenmeyer flask is filled with 50 mL of sodium hydroxide solution (concentrations 0.2 and 1 M) and 5 g of pure zeolite. For two hours, the mixture is vigorously agitated at a temperature of 80 °C. Following, the collected sediment is washed with deionized water, and the supernatant liquid is decanted. The rinsing process is repeated until the pH of the water becomes approximately neutral. The zeolite is removed and left on a petri dish to air-dry at 60 °C for 24 h. In a porcelain pestle, dry alkali-treated zeolite is ground and powdered. The additives produced from the treatments are presented in Table 2.

Table 2. Modified Bentonite and Zeolite 13X.

Treatment	Bentonite	Zeolite 13X
None	Be	13X
Alkali, 0.2 M NaOH solution	No Treatment	13X02
Alkali, 1 M NaOH solution	Be1	13X1

2.2. Anaerobic Experimental Process

2.2.1. Feedstock and Inoculum

The inoculum originated from a full-scale mesophilic (37 ± 1 °C) biogas plant in northern Greece and was preincubated under anaerobic condition at 37 ± 1 °C for 4 days to reduce its residual CH₄ production. Cattle manure was used as substrate and was provided by a dairy farm in the Lagada region, Thessaloniki, Greece. The characteristics of the inoculum and the substrate are presented in Table 3, and each measurement was performed in triplicate.

Table 3. Inoculum and substrate characteristics.

Parameter	First Set		Second Set	
	Inoculum (Mean \pm SD *)	Substrate (Mean \pm SD *)	Inoculum (Mean \pm SD *)	Substrate (Mean \pm SD *)
Total Solids, TS (g L ⁻¹)	22.79 \pm 3.31	64.99 \pm 5.26	34.06 \pm 2.01	65.24 \pm 4.53
Volatile Solids, VS (g L ⁻¹)	14.56 \pm 1.73	56.75 \pm 2.82	25.03 \pm 1.68	58.31 \pm 3.52
Total Ammonia Nitrogen, TAN (mg N L ⁻¹)	1040 \pm 11.65	1800 \pm 45.84	1680 \pm 25.78	1960 \pm 31.55
Volatile Fatty Acids, VFA (g L ⁻¹)	1.83 \pm 0.06	39.94 \pm 0.74	2.63 \pm 0.08	46.58 \pm 0.95
Total Sulfur, TSf (mg kg ⁻¹)	75.58 \pm 1.94	83.59 \pm 5.11	82.80 \pm 2.34	86.00 \pm 3.59
pH	7.63 \pm 0.08	7.71 \pm 0.05	7.75 \pm 0.06	7.84 \pm 0.03

*SD, Standard Deviation.

2.2.2. Experimental Setup

Two sets of experiments were carried out for the purposes of this study. The effect of the modified additives was tested in AD under high ammonia concentration conditions (1st set of experiments) and under high ammonia and sulfate concentration conditions (2nd set of experiments). Glass bottles with a total volume of 321 mL and an operating volume of 150 mL were used as batch reactors. The batch reactors were filled with inoculum and cattle manure, in order to achieve a manure-to-inoculum ratio of 1 in terms of volatile solids (VS) [19], and the rest was filled with deionized water to reach the operating volume of 150 mL. Blank reactors were filled only with inoculum and deionized water up to the same operating volume. The concentration of the inorganic additives in the reactors was 8 g L⁻¹, as this concentration has shown previously improved reactor performance [20]. To ensure anaerobic conditions, all batch reactors were flushed with a gas mixture of N₂/CO₂ (80/20, v/v) and were sealed with rubber stoppers and aluminum caps. The reactors were incubated at 37 ± 1 °C and vigorously mixed, manually, once per day.

For the first set, 24 batch reactors were prepared corresponding to 2 different treatments of bentonite, 3 treatments of zeolite 13X, 2 different controls for reference and a blank in order to monitor the CH₄ production of the inoculum and subtract it from the production of the batch reactors. Three (3) replications were prepared for all treatments. The contents of each treatment and their respective names are presented in Table 4. In order to achieve NH₃ inhibition conditions in the reactors, the NH₃ concentration was set at 4500 mg L⁻¹, with the addition of ammonium chloride (NH₄Cl, Sigma-Aldrich, Burlington, MA, USA, purity 99.998%).

Table 4. First set of batch reactors.

Treatment	Additive (g/Reactor)	Inoculum (g VS/Reactor)	Substrate (g VS/Reactor)	TAN (mg L ⁻¹)
PBe	1.2	1	1	4500
PBe1	1.2	1	1	4500
P13X	1.2	1	1	4500
P13X02	1.2	1	1	4500
P13X1	1.2	1	1	4500
PCNH ₃	0	1	1	4500
PC ₀	0	1	1	910
PBlank	0	1	0	693

For the second set, 90 batch reactors were prepared corresponding to 2 different treatments of bentonite, 3 treatments of zeolite 13X, 3 different controls for reference and a blank in order to monitor the CH₄ production of the inoculum and subtract it from the production of the batch reactors. Nine (9) replications were prepared for all treatments. The contents of each treatment and their respective names are presented in Table 5. In order to achieve NH₃ and H₂S inhibition conditions in the reactors, the NH₃ and sulfate (SO₄²⁻) concentrations were set at 3500 mg L⁻¹ and 2270 mg L⁻¹, respectively [5,21], with the addition of ammonium chloride (NH₄Cl, Sigma-Aldrich, Burlington, MA, USA, purity 99.998%) and sodium sulfate (Na₂SO₄, Lach-Ner, Neratovice, Czech Republic, purity 99.2%). The use of Na₂SO₄ is a common method to increase the SO₄²⁻ concentration in anaerobic batch experiments [22].

Table 5. Second set of batch reactors.

Treatment	Additive (g/Reactor)	Inoculum (g VS/Reactor)	Substrate (g VS/reactor)	TAN (mg L ⁻¹)	SO ₄ ²⁻ (mg L ⁻¹)
Be	1.2	1	1	3500	2270
Be1	1.2	1	1	3500	2270
13X	1.2	1	1	3500	2270
13X02	1.2	1	1	3500	2270
13X1	1.2	1	1	3500	2270
CNH ₃ -SO ₄	0	1	1	3500	2270
CNH ₃	0	1	1	3500	N.D. *
CSO ₄	0	1	1	680	2270
C ₀	0	1	1	680	N.D. *
Blank	0	1	0	450	N.D. *

* N.D., Not Detected.

2.3. Analytical Methods

2.3.1. Physicochemical Analyses for the Anaerobic Process

Total solids (TS), volatile solids (VS), total sulfur (TSf) and total ammonia nitrogen (TAN) were determined in accordance with standard methods [23]. Measurements of the pH value of the solutions were performed with a bench digital pH meter (JENWAY 3520, Essex, UK). The volatile fatty acid (VFA) samples were analyzed with a gas chromatograph (GC-2010plusAT, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID). Biogas samples were obtained daily from the headspace of the reactors with a gastight syringe equipped with a pressure lock and an attached needle. The gas samples were injected into a gas chromatograph (GC-2010plusAT, SHIMADZU, Kyoto, Japan) equipped with a thermal conductivity detector (TCD), and biogas composition was determined [21].

The Agilent 7850 ICP-MS (Agilent Technologies, Waldbronn, Germany) mass spectrometer was used for the measurement of trace elements. The method of Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) that was followed is outlined in ISO 17294-2:2016. First, 0.5–1.0 g of the initial sample is weighed and mixed with nitric acid (HNO₃) and

hydrogen peroxide (H₂O₂). The mixture is then decomposed in a high-pressure digestion vessel. The resulting solution is analyzed using ICP-MS.

2.3.2. Determination of Ammonia and Sulfate Removal Capacity

To determine the inorganic additives' ARC, 5 g of inorganic additive are set in a falcon tube with a capacity of 50 mL, and a solution of ammonium chloride with a concentration of 5000 mg L⁻¹ was poured up to the mark. The falcon tubes are kept incubated for 24 h at 37 °C. They are then centrifuged, with the liquid supernatant being decanted and filtered. In 25 mL Erlenmeyer flasks, 25 mL of the sample, 3 drops of Mineral Stabilizer reagent, 3 drops of Polyvinyl Alcohol Dispersing Agent reagent, and 1 mL of Nessler Reagent were added, and the final solution is stirred. The same procedure is also followed in order to determine the inorganic additives' sulfate removal capacity (SRC), but a solution of sodium sulfate with a concentration of 2300 mg L⁻¹ was used. From the resulting supernatant liquid, 50 mL are placed in a 250 mL Erlenmeyer flask and are vigorously stirred. While stirring, 10 mL of buffer solution and 1 mL of 10% BaCl₂ solution are added. The flask is removed from the stirrer after 60 s of continuous stirring and left to rest for 4 min. The respective supernatants NH₄⁺ or SO₄²⁻ concentration is calculated from the absorbance measured at 420 nm with an optical path of at least 1 cm using a JASCO V-630 (Lightpath Optical Ltd., Axminster, UK) Spectrophotometer and quartz cells, with a route of 10 mm.

The inorganic additive's ARC and SRC are calculated according to the following formula:

$$RC = \frac{(C_O - C_S) \times V_{S_0}}{W_{IA}} \quad (1)$$

where:

RC: removal capacity (mg g⁻¹ of inorganic additive);

C_O: concentration of the initial solution (mg L⁻¹);

C_S: concentration of the supernatant (mg L⁻¹);

W_{IA}: mass of the inorganic additive (g);

V_{S₀}: volume of the initial solution (L).

2.3.3. Physicochemical Analyses of Modified Additives

The Autosorb-1-MP (Quantachrome, Boynton Beach, FL, USA) adsorption analyzer was used to obtain the N₂ adsorption–desorption isotherms (77 K) and extract the pore structural properties including the BET surface, the micropore and mesopore volume and the pore size distributions. For the latter, the Non-Local Density Functional Theory (NLDFIT) method for silica and N₂ as adsorbate was applied to interpret both the adsorption (adsorption model-cylindrical pores) and desorption (equilibrium model-cylindrical pores) branches of the isotherm. Before the measurement, all samples were outgassed at high-temperature (300 °C) and high-vacuum conditions (10⁻⁵ mbar) for 24 h.

A Jeol JSM-7401F (Jeol, Tokyo, Japan) Field Emission Scanning Electron Microscope equipped with Gentle Beam mode was employed to characterize the surface morphology of the samples. Gentle Beam technology can reduce charging and improve resolution, signal-to-noise and beam brightness. Scanning electron microscopy (SEM) was performed at typical conditions of 10 mA of emission current and 2 kV of accelerating voltage. Samples were mounted on metallic (brass) substrates using a double-coated carbon conductive tape. Elemental mapping distributions of Al, Si, Na, Mg, Fe, Cu, Ca, K and O were acquired by EDS microanalysis, at an acceleration voltage of 20 kV, using an Xplore-15 SDD detector (Oxford Instruments, Abingdon, UK) with a surface of 15 mm².

2.3.4. Statistical Analyses

Data for the two sets of experiments were analyzed with the ANOVA method within the methodological frame of General Linear Models. The ANOVA method was used mainly for estimating the correct standard errors of the differences among mean values compared with the Tukey's multiple comparisons procedure [24]. Linear models' residuals were

tested for normality and homoscedasticity. No serious violations of these two assumptions were detected. In addition, the reliability of the experiments was high since the corresponding Coefficient of Variation (CV) values were very small. Data are reported as mean values \pm standard deviation. In all hypothesis testing procedures, the significance level was preset at $\alpha = 0.05$ ($p \leq 0.05$). All statistical analyses were accomplished with the IBM SPSS Statistics v.23.0 software.

3. Results and Discussion

3.1. Characteristics of Additives

In Table 6, the physicochemical properties of the additives are presented. Alkali treatment of zeolite 13X with a 0.2 M solution of NaOH led to an increase in BET surface area, corresponding with the formation of micropores [25]. The use of a 1 M solution caused the collapse of the zeolite's micropores, causing an overall decrease in surface area but the increase in the mesopore volume; similar results have been observed by Ates and Akgül [26]. Furthermore, the treatment of zeolite with increasing concentrations of NaOH solutions lowered both its NH_3 and its SO_4^{-2} adsorption capacity. A possible explanation is that NaOH treatment alters the zeolite's acid site distribution, significantly replacing zeolitic protons with sodium cations [27]. The composition of zeolite 13X was altered after the alkali treatment, the use of both 0.2 M and 1 M NaOH solutions led to a slight increase in sodium in the zeolite's matrix by lowering the oxygen, silica and alumina concentrations and replacing potassium completely.

Table 6. Physicochemical Properties of Inorganic Additives.

Property	Be	Be1	13X	13X02	13X1
NH_3 Adsorption Capacity (mg g^{-1})	10.9	23.25	34.076	31.53	28.97
SO_4^{-2} Adsorption Capacity (mg g^{-1})	38.51	28.14	53.32	28.14	1.48
BET specific surface ($\text{m}^2 \text{g}^{-1}$)	52	13	688	969	110
Micropore volume (mL g^{-1})	N.D. *	N.D. *	0.35	0.485	0.036
Mesopore volume (mL g^{-1})	0.150	0.086	N.D. *	N.D. *	0.340

* Not Detected.

Bentonite that is subjected to alkali treatment generally improves its NH_3 adsorption capacity [28]. The reaction of bentonite with alkaline solutions produces hydrous hydroxy-aluminosilicate and hydrous carbonate mineral phases that can result in pore filling, reducing the BET surface of the bentonite [29].

3.2. Effect of Modified Additives on Methane Production under High Ammonia Concentration

The effect of high ammonia concentration on the anaerobic process was evaluated by comparing treatment PC0 (without additives addition and with initial TAN concentration at 910 mg L^{-1}) with treatment PCNH3 (without additives addition and with initial TAN concentration at 4500 mg L^{-1} due to NH_4Cl introduction). In Figure 1b, the accumulative CH_4 production per day for each treatment is given. As can be seen during the first days of the incubation period, a strong inhibitory effect of the ammonia on the methane production was observed in treatment PCNH3 in comparison with PC0. A prolonged lag phase of the methanogens growth is an indicator of ammonia inhibition corresponding with findings by Fotidis et al. [30,31]. After this period, the methane production rate was gradually increased, which denotes acclimatization of the methanogens to high ammonia concentrations [21], and finally the methane production reached $243.2 \text{ mL per g of VS}$ at the end of the incubation period (ca. 8% less than PC0, but no statistically significant $p < 0.05$). Given the appropriate time to adapt, methanogens are known to become acclimatized to high ammonia concentrations, allowing them to overcome ammonia toxicity conditions [32]. The addition of zeolites and other clay minerals can have a beneficial or detrimental effect on the AD process, depending, among other factors, on their concentration [33–35]. Regarding the treatments containing inorganic additives, all of them showed improved

performance compared to treatment PCNH3 (Figure 1a), and none of them showed any negative effect on the anaerobic process. These results in conjunction with the relatively low VFA concentrations in all the treatments (Figure 2) indicate that, despite the high concentration of ammonia (4500 ppm) in the treatments where NH_4Cl was added, the ammonia toxicity was mild in the anaerobic process and that the additives improved the methane production. In the bentonite treatments, the final pH values were relatively higher than in the zeolite 13X treatments (Table 7).

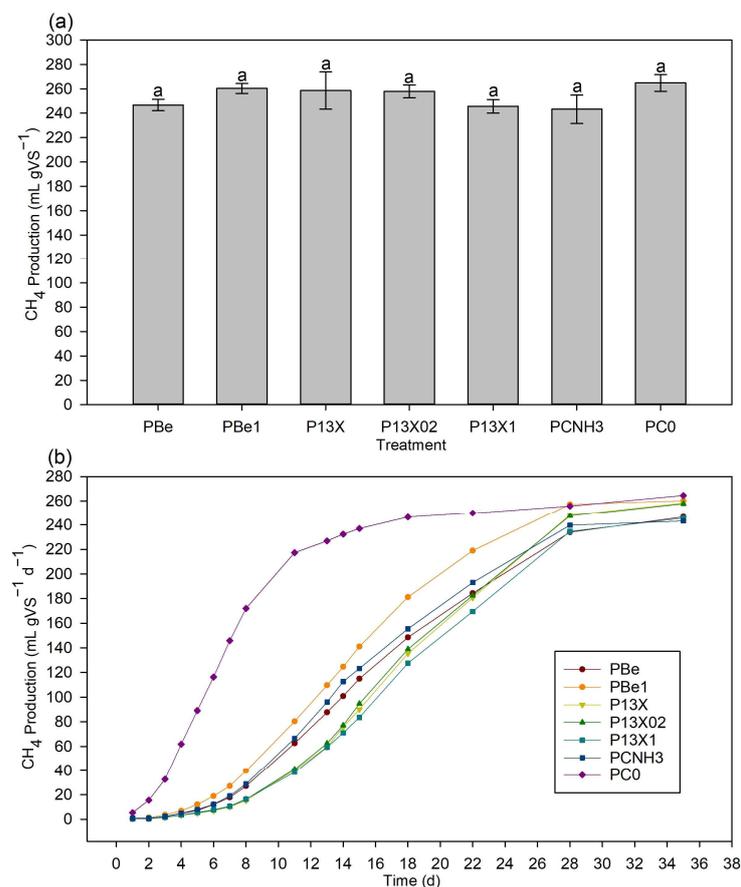


Figure 1. (a) Total methane yields and (b) accumulative biogas production under high ammonia concentration. In (a), bars with the same letter “a” above them correspond to mean values and are not statistically significantly different, at $\alpha = 0.05$ ($p > 0.05$), according to the results of the Tukey’s test. Error bars correspond to \pm Standard Deviation. (Summary results of ANOVA $p > 0.05$, $R^2 = 0.557$, $CV = 3.4\%$).

Table 7. Final pH values under high ammonia concentration.

Treatment	pH (Mean \pm SD *)
PBe	7.74 \pm 0.20
PBe1	7.81 \pm 0.13
P13X	7.64 \pm 0.21
P13X02	7.39 \pm 0.28
P13X1	7.22 \pm 0.22
PCNH3	7.23 \pm 0.42
PC0	7.75 \pm 0.32

* SD, Standard Deviation.

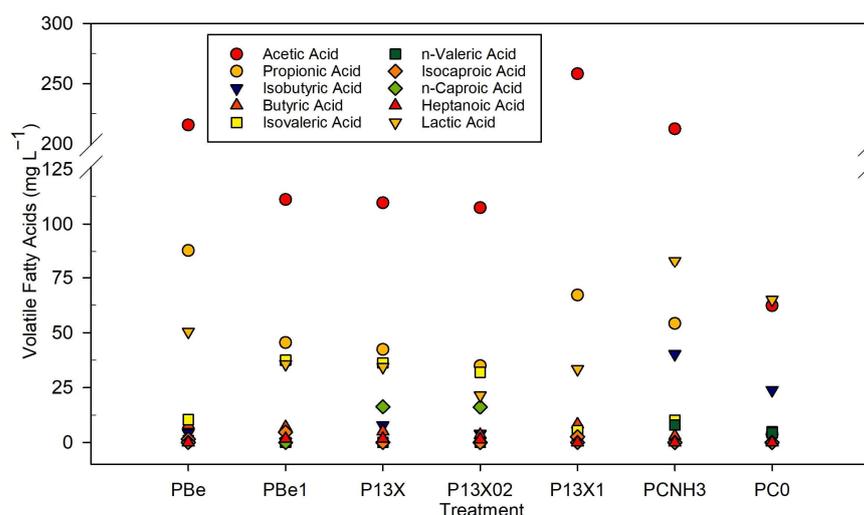


Figure 2. Total VFA accumulation under high ammonia concentration.

Anaerobic Process under High Ammonia and Sulfate Concentrations Toxicity Effect of Sulfate and Ammonia on Methane Production

In Figure 3a, the methane reduction caused by the presence of ammonia and sulfate individually as well as in the copresence of both of them in comparison with the blank—C0 (where no inhibitors are added)—is illustrated. As can be seen, a slight decrease in methane production in treatment CNH3 by 3.99% (but not statistically significant, $p > 0.05$) was observed due to the ammonia addition, like the first set of the experiment. A drastic reduction in methane production was observed in the treatments where sulfate was added. Specifically, the total methane produced by treatment CSO4, where sulfate was the sole inhibitor, was $170.52 \text{ mL gVS}^{-1}$, significantly reduced by 29.7% ($p < 0.05$) than that of C0. This indicates that the addition of sulfate introduces an important inhibition factor on methane production in our case. Sulfate is reduced to sulfide by sulfate-reducing bacteria (SRB); SRB, due to their more favorable growth and thermodynamic properties against methanogens and acetogens, outcompete them for common substrates such as H_2 and other organic substances, resulting in reduced methane production [36]. In accordance with this study, the inhibitory effects of excess sulfate on the methane production have also been reported elsewhere [37]. It was also reported that dissolved sulfide in the range of $100\text{--}800 \text{ mg L}^{-1}$, which is produced by the SRB, can cause inhibition to various trophic groups involved in the anaerobic process, which adversely affects the methane production [6]. In treatment CNH3-SO4, with high ammonia and sulfate concentrations, the methane production was further decreased ($156.47 \text{ mL gVS}^{-1}$), resulting in a statistically lower production by 8.24% and 35.45% compared to CSO4 and C0, respectively. Hansen et al. have discovered a synergistic inhibitory effect of ammonia and sulfide on methane production, which can potentially explain the significant decrease of methane production in treatment CNH3-SO4 [16].

The accumulative methane production curves are presented in Figure 3b. The curve of treatment CSO4 is of great interest, because the methane production does not seem to be affected by the high sulfate concentration during the first days of the experiment, since it follows the same trend as C0. This further supports that the direct inhibition of methanogenesis by sulfate is minor but causes indirect inhibition by promoting the growth of SRB that outcompete methanogens, resulting in reduced methane production, as aforementioned [38]. After the first 8 days, a sudden dramatic decrease was observed in the daily methane production of CSO4, probably as a result of the inhibition caused by the hydrogen sulfide produced and the antagonistic growth of SRB against the methanogenic cultures. While initially the methane production of treatment CNH3 was low, it grew steadily and eventually exceeded that of CSO4 after the first days of the experiment, suggesting that there was a gradual acclimatization of the methanogenic microorganisms to high ammonia concentrations. The low methane production rate throughout the duration of the exper-

iment in the CNH3-SO4 treatment shows the synergistic action of the two inhibitors in the anaerobic process. VFAs are presented in Figure 4 and pH in Table 8. As can be shown with increased toxicity, VFAs were increased and pH showed a relative decreasing trend accordingly.

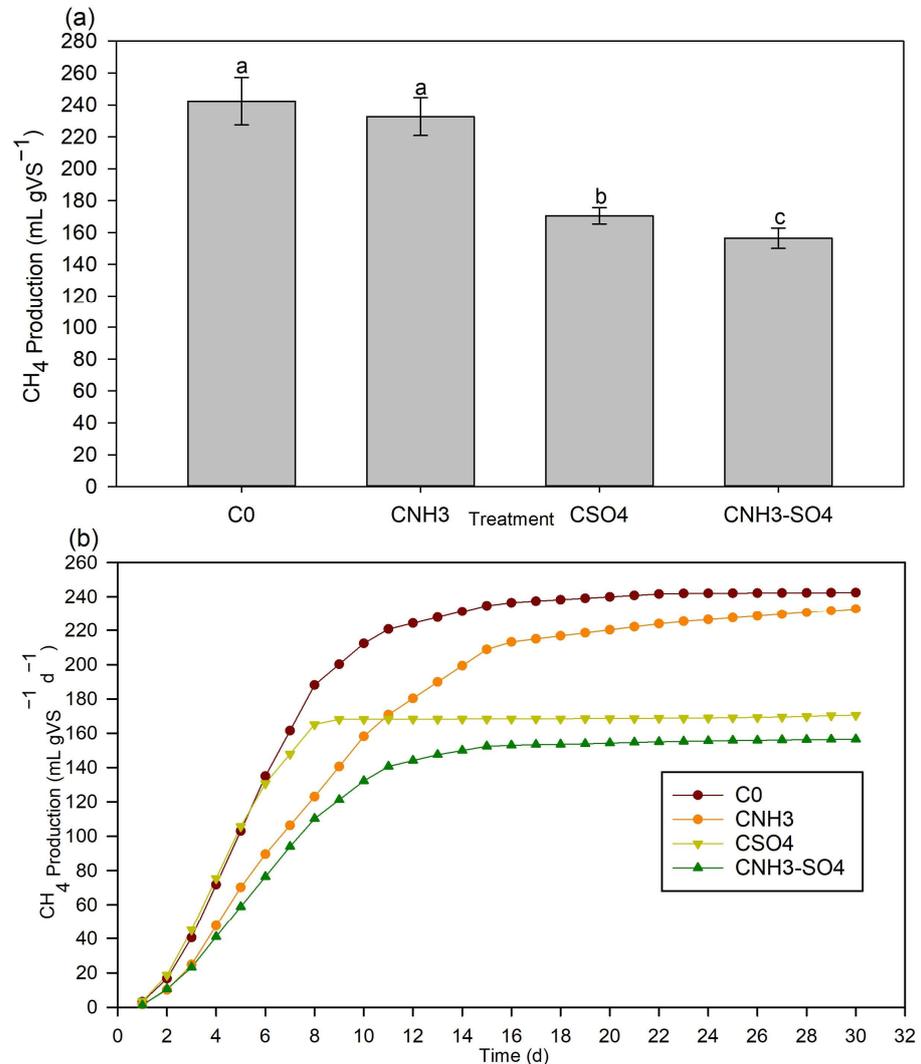


Figure 3. (a) Total methane yields and (b) accumulative biogas production under high ammonia and sulfate concentrations. In (a), different letters above the bars correspond to mean values that are statistically significantly different, at $\alpha = 0.05$ ($p < 0.05$), according to the results of the Tukey's test. Error bars correspond to \pm Standard Deviation. (Summary results of ANOVA $p < 0.001$, $R^2 = 0.937$, $CV = 5.2\%$).

Table 8. Final pH values under high ammonia and sulfate concentrations.

Treatment	pH (Mean \pm SD *)
C0	7.82 \pm 0.45
CNH3	7.61 \pm 0.36
CSO4	7.42 \pm 0.20
CNH3-SO4	7.36 \pm 0.62

*SD, Standard Deviation.

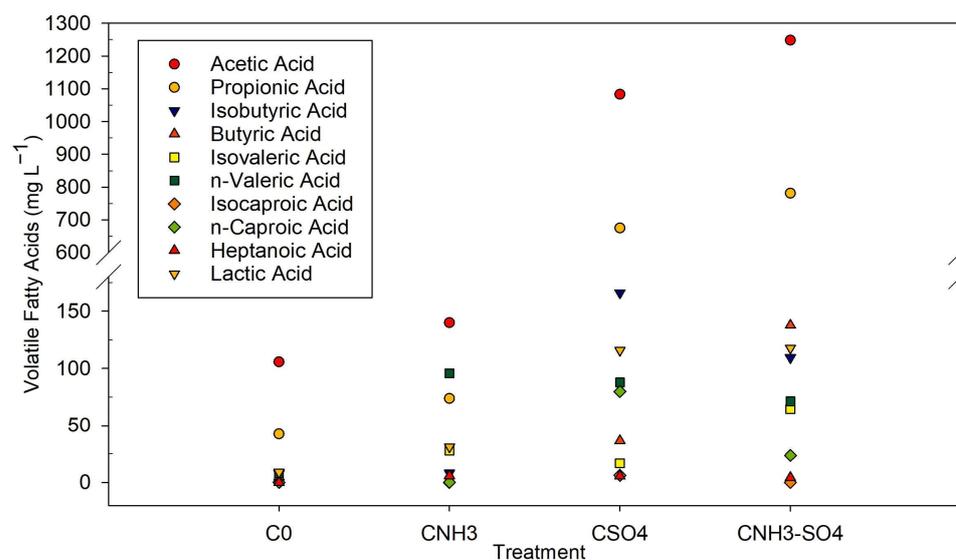


Figure 4. Total VFA accumulation under high ammonia and sulfate concentrations.

Effect of the Modified Additives on the Anaerobic Process under High Ammonia and Sulfate Concentrations

The accumulative methane production of the additive treatments is presented in Figure 5b. The accumulative methane production curve of all the treatments followed the same trend during the first 9–10 days of the experiment. The trends started to differentiate mainly after day 15, when in the Bentonite treatments (Be, Be1), the daily methane production became limited or nonexistent, showing similarities with that of treatment CNH3-SO4. Meanwhile, in the zeolite 13X treatments (13X, 13X02, 13X1), it kept increasing, indicating that the addition of zeolite 13X was beneficial for the whole duration of the experiment.

The total methane production per g of VS for the additive treatments compared with that of CNH3-SO4 is presented in Figure 5a. The addition of zeolite 13X reduced the inhibitory action of ammonia and sulfate, enhancing the methane production yield significantly ($p < 0.05$) compared to the control treatment CNH3-SO4, by 8.96%, 11.00% and 15.56% in treatments 13X, 13X02 and 13X1, respectively. Interestingly, the addition of bentonite did not lead to similar results; it either did not improve (Be) or caused a slight increase (Be1) in methane production compared to CNH3-SO4, despite the fact that bentonite treatments demonstrated their capacity to adsorb sulfate at higher degrees than the zeolite 13X1 (Table 6), whose treatment had the best results for methane production. This result suggests that sulfate adsorption in the liquid phase is not likely to be the mechanism for inhibition reduction. It has previously been reported that zeolite 13X has the ability to adsorb at its surface sufficient quantities of hydrogen sulfide in leachate wastewaters, which probably occurred in our case as well, improving the anaerobic degradation in the treatments with zeolite 13X [15]. Another possible explanation for the positive effect exhibited by zeolite 13X could be that 13X particles can offer an immobilization matrix for methanogens, and it was found that zeolites have a high capacity for microorganisms immobilization [34] and could provide specific favorable conditions for the adherence of microbial consortium, resulting in an enhanced anaerobic process [39]. SEM images of the bentonite and zeolite particles are presented in the Supplementary Materials (Figure S5).

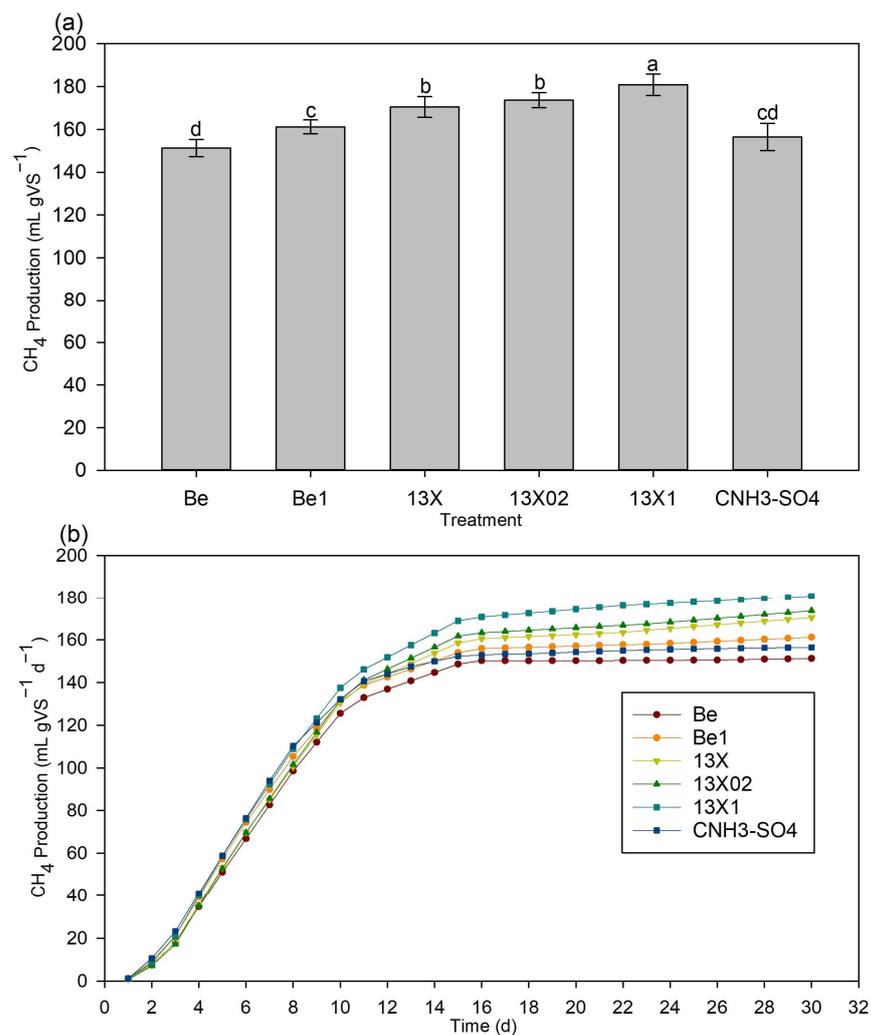


Figure 5. (a) Total methane yields and (b) accumulative biogas production under high ammonia and sulfate concentrations. In (a), different letters above the bars correspond to mean values that are statistically significantly different, at $\alpha = 0.05$ ($p < 0.05$), according to the results of the Tukey's test. Error bars correspond to \pm Standard Deviation. (Summary results of ANOVA $p < 0.001$, $R^2 = 0.847$, $CV = 2.8\%$).

These explanations cannot adequately account for the large difference in toxicity reduction shown by the 13X zeolite versus bentonite treatments. We looked further into the concentration of cations in the aqueous phase of the reactors (Figure 6). It was evident that there was great differentiation in the sodium concentration between the zeolite 13X and bentonite treatments. Specifically, the sodium concentration in zeolite 13X treatments (mean value ~ 2390 mg kg⁻¹) is 40% higher than that of the bentonite treatments (mean value ~ 1710 mg kg⁻¹). These results indicate that the higher sodium concentrations observed in zeolite 13X treatments could have led to the increase in methane production compared to the bentonite treatments with the lower sodium concentration. It has been reported that sodium concentrations higher than 3.5 g L⁻¹ can cause moderate inhibition of the methanogenesis [6]; then again, studies show that the addition of large quantities of sodium hydroxide (NaOH) and sodium bicarbonate (NaHCO₃) increases significantly the methane production during AD [40,41], which was mainly attributed to pH regulation. It has also been found that sodium concentrations of 4.42 g L⁻¹ enhanced the methane yield compared to 0.49 g L⁻¹ in the anaerobic digestion of *Sargassum* sp., resulting in methane production of 290.41 instead of 210.29 mL CH₄ g VS⁻¹ [42]. Furthermore, it has been observed that at 2.6 g L⁻¹ sodium concentration, a similar concentration with

the 13X treatments, the anaerobic digestion was improved in the presence of high sulfide (836 mg L^{-1}) and ammonia (4.5 g L^{-1}) concentrations, compared to sodium concentrations of 1 or 4 g L^{-1} [43]. In the current case, there is a direct correlation between the increase in methane production of the 13X treatments and the higher sodium concentration that was observed. In the bentonite treatments, increased concentrations of calcium and manganese were also observed, both of which can possibly act competitively with sodium limiting its toxicity alleviation effect [44]. It must be noted that the positive results of 13X could be affected by the relatively increased pH value compared to that of the bentonite (Table 9), which was conducive to the transformation of the unionized sulfide fraction in the aqueous phase, that is the most toxic to the methanogens, to hydrosulfide (HS^-) [44].

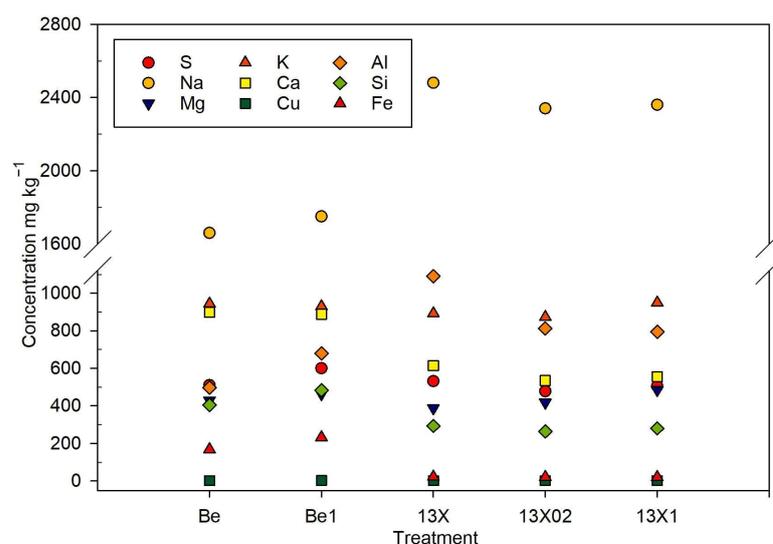


Figure 6. Concentration of cations in the aqueous phase of the reactors under high ammonia and sulfate concentrations.

Table 9. Final pH values under high ammonia and sulfate concentrations.

Treatment	pH (Mean \pm SD *)
Be	7.53 \pm 0.60
Be1	7.64 \pm 0.35
13X	7.76 \pm 0.31
13X02	7.69 \pm 0.47
13X1	7.81 \pm 0.13
CNH3-SO4	7.36 \pm 0.62

* SD, Standard Deviation.

The aforementioned hypothesis coincides with the increased beneficial effect of zeolite 13X treated with higher concentrations of NaOH solutions (Figure 5a). According to the supplementary data (Figures S3 and S4), the treatments with higher NaOH concentrations resulted in increased sodium content for 13X02 and 13X1 compared to the unmodified zeolite 13X, which led to increased methane production over the unmodified 13X. While, the further increase in methane production observed in 13X1 compared to 13X02 treatment can be attributed to its larger mesoporous structure (Table 6), which permits the faster migration of guest molecules in the host frameworks than the micropores, that are more abundant in 13X02 [45]. In this study, it has been proven that with NaOH treatment, zeolite 13X can counteract to a degree the simultaneous inhibition of sulfate and ammonia; however, targeted analyses and the investigation of cation interactions as well as the microbial functions are needed to further understand the mechanism behind this.

The concentrations for the individual VFAs are presented in Figure 7, where it is observed that the decrease of VFA concentration coincides with the increase in methane

production across all the treatments. VFAs are key intermediates in AD, and with the uninhibited conclusion of the anaerobic process, the concentration of VFAs should be negligible, as they should be converted to methane and carbon dioxide. Their presence in the aqueous phase, and especially the presence of propionic acid, indicates an imbalance in the process [46]. The relatively high concentration of propionic acid in treatments Be and Be1 indicates that the addition of bentonite has not counteracted the ammonia and sulfate toxicity.

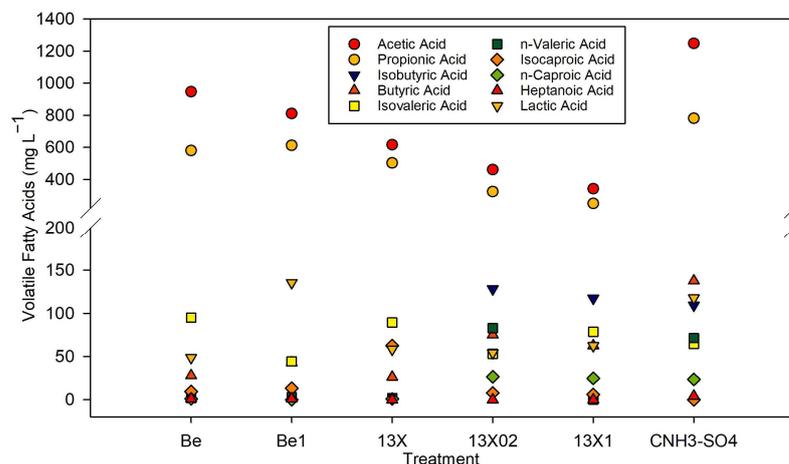


Figure 7. Total VFA accumulation under high ammonia and sulfate concentrations.

4. Conclusions

A co-inhibitory effect on methane production in the presence of ammonia and sulfate was observed. Under high ammonia and sulfate concentrations, the biomethane performance was improved in anaerobic batch reactors by the addition of zeolite 13X, a clear sign that 13X was able to alleviate ammonia and sulfate co-derived toxicity. The increase in methane production was greater for zeolite 13X additives that were treated with higher NaOH concentrations, indicating that the Na^+ cation, among other parameters, plays an important role in this alleviation. Bentonite unmodified or modified with NaOH did not effectively mitigate the toxicity. These findings could potentially be exploited by the biogas plant operators to improve their biomethane performance when operating under high ammonia and sulfate concentrations, after an economic feasibility investigation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/su15139994/s1>, Figure S1: SEM micrograph of unmodified bentonite sample and the corresponding elemental maps.; Figure S2: SEM micrograph of unmodified zeolite 13X sample and the corresponding elemental maps.; Figure S3: SEM micrograph of zeolite 13X treated with 0.2 M NaOH sample and the corresponding elemental maps.; Figure S4: SEM micrograph of zeolite 13X treated with 0.1 M NaOH sample and the corresponding elemental maps.; Figure S5: Low- and high-magnification SEM images of the pristine and NaOH-treated samples showing the quite different morphological characteristics of the Bentonite and Zeolite 13X particles. (a, b and g, h) Low- and high-magnification SEM of the pristine 13X and Bentonite, respectively. (c, d) Low- and high-magnification SEM of the 1 M NaOH-treated 13X. (e, f and I, j) Low- and high-magnification SEM of the 0.2 M NaOH-treated 13X and Bentonite, respectively. Comparison between Figures b, d and f show that the denser NaOH solution starts to erode the surface of the 13X crystal, making visible the existence of surface roughness.; Table S1: First set: daily methane production (Mean \pm SD*), Table S2: Second set: daily methane production (Mean \pm SD*).

Author Contributions: Conceptualization, T.A.K. and C.A.T.; methodology, C.A.T. and S.D.K.; software, G.C.M.; investigation, C.A.T., C.M.V., E.-A.E. and G.E.R.; writing—original draft preparation, C.A.T.; writing—review and editing, S.D.K., E.-A.E., G.E.R., A.M., G.C.M., T.S. and T.A.K.; supervision, T.A.K.; project administration, T.S. and T.A.K.; funding acquisition, T.A.K. and T.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was co-financed by the European Regional Development Fund of the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH—CREATE—INNOVATE (project code: T2EDK-00359).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: This research was co-financed by the European Regional Development Fund of the European Union and Greek national funds through the Operational Program Competitiveness, Entrepreneurship and Innovation, under the call RESEARCH—CREATE—INNOVATE (project code: T2EDK-00359).

Conflicts of Interest: The authors declare no conflict of interest.

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