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Enhancing sludge biodegradability through free nitrous acid pre-treatment at low exposure time

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Abstract

The effectiveness of low free nitrous acid (FNA) pre-treatment times (PTs) (<8 h) on waste activated sludge (WAS) is not known. This study explores the effectiveness of four different FNA concentrations (0, 2.49, 3.55 and 4.62 mg N-HNO_2/L) and three low PTs (2, 5 and 8 h) on WAS characteristics and methane generation. Increasing FNA concentrations and PTs resulted in an increase in the solubility of the organic matter (chemical oxygen demand, proteins and polysaccharides). Cell viability was below 15% in all cases at PTs higher than 2h. Biochemical methane production tests (BMP) showed a significant increase (20-27 %) on specific and total methane production (SMP and MP) when the sludge was pretreated with 2.49- and 3.55 mg N-HNO_2/L during 5 h and 8 h. Increasing PT (>5 h) resulted in a decrease in MP due to a volatile solid reduction on WAS during the pretreatment. The highest FNA concentration tested (4.62 mg N-

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HNO_2/L) did not further improve MP and SMP. This study clearly shows the effectiveness of the FNA sludge pretreatment at low exposure times.

Keywords: anaerobic digestion, free-nitrous acid pre-treatment, methane production, secondary sludge biodegradability, waste activated sludge.

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1. Introduction

It is well known that methane production (MP) from waste activated sludge (WAS) is often limited by the slow fermentation rates (hydrolysis and acidification) and its poor biochemical methane potential (BMP)[1–10]. To overcome this, recent research has been focusing on developing a novel, attractive and economic pre-treatment for WAS based on free nitrous acid (FNA) [6,7,11–14]. FNA destroy cells and solubilize the extracellular polymeric substances (EPS) (especially proteins and polysaccharides) present in WAS, causing the release of intracellular and/or extracellular constituents to the aqueous phase [4,6,7,11,15], which are more easily biodegradable during anaerobic digestion (AD), thereby enhancing specific methane production (mL of methane per volatile solid added) (SMP) [6]. FNA pre-treatment has been demonstrated to cause a strong biocidal impact on microorganisms, to increase sludge biodegradability and SMP at FNA concentrations in the range of 0.36-2.13 mg N- HNO_2/L [6,7,12,13]. FNA exists in equilibrium with nitrite and the mechanisms by which nitrite and FNA have been reported to act as cytotoxins include the following [16,17]*:* (i) FNA can lead to the formation of reactive nitrogen and oxygen species in the cytoplasm including nitric oxide (NO), nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), hydroxide ion (OH^-) and hydrogen peroxide (H_2O_2), all of which exhibit toxicity towards bacterial cells, causing cell death; (ii) FNA has been suggested to act as an uncoupler acting to circumvent the ATP synthesis as a result of a short-circuit formed by FNA transporting protons across the inner membrane and back into the cell and so increasing the conductance of the cytoplasmic membrane; and (iii) FNA may be able to directly inhibit electron carriers. The disadvantage of the FNA pre-treatment is the long pretreatment times (PTs) suggested in the literature to date (24 h). This is an important aspect, because a reduced PT is preferred for real application since it reduces the volume of the pretreatment tank.

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With this study we aim at exploring the effect of pre-treatment times lower than the ones reported in literature (24 h) on WAS biodegradability and methane generation. The effect of the FNA pre-treatment on the sludge characteristics was assessed at 4 different FNA concentrations (0, 2.49, 3.55 and 4.62 mg N-HNO_2/L) and 3 different exposure times (2, 5 and 8 h). To evaluate the effect of the different FNA pre-treatment on the biochemical methane potential (BMP), BMP tests were conducted in triplicates for each conditions tested. This is the first study reporting the effectiveness of the FNA pre-treatment in WAS at low pre-treatment times and on MP (not only on the SMP). This is important, since for industrial application, the increase on the SMP have to be linked to an increase on the MP and not due to a volatile solid reduction on WAS during the pre-treatment. Until now the FNA biocidal effect on SMP of WAS was only associated with the FNA concentration but not with the PT.

2. Materials and methods

2.1 Lleida WWTP, WAS and Inoculum

The Lleida WWTP (Catalonia, Spain) where the sludge and inoculum was taken serves 130,000 PE treating 60,000 m^3/d of domestic wastewater. The conventional treatment used in this plant consists of preliminary (fat and sand removal equipment with a hydraulic retention time (HRT) of 12 min), primary treatment (primary clarifier with HRT=1.82 h), and then a secondary treatment where microorganisms are used to consume organic matter and nutrients from the wastewater in the activated sludge unit (HRT and solid RT (SRT) of 0.36 d and 9.55 d, respectively). Primary sludge (PS) is a result of capturing suspended solids and organics in the primary treatment process through gravitational sedimentation and WAS is obtained from the secondary clarifier (HRT=4.88 h). PS and secondary WAS are thickened and mixed (sampling point) before undergoing mesophilic (37 ºC) anaerobic digestion at 26 days of SRT in two

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anaerobic bioreactors with a total volume of 5000m^3/d and biogas production around 2800 m^3/d. Finally, digested sludge is dewatered before its disposal.

For the present study, WAS was collected from the secondary sludge thickener. The pH, total solids (TS), volatile solids (VS) and soluble chemical oxygen demand (SCOD) concentrations were 6.4±0.1, 40±1 g TS/kg, 29±0 g VS/kg and 51.6±0.2 mg SCOD/g VS, respectively.

For the BMP tests, the inoculum was collected from one of the mesophilic anaerobic digester present at the same WWTP. This digester has a capacity of 2480 m^3 and treats mixed sludge produced in the WWTP. The pH, TS, VS and SCOD concentrations in the inoculum were 7.8±0.2; 25±0 g TS/kg, 14±0 g VS/kg and 24.9±0.2 g SCOD/g VS, respectively.

2.2. FNA pre-treatment methodology

The FNA pre-treatment tests were carried out in four polyethylene batch reactors of 1.5 L of working volume. The batch reactors were covered during the pretreatment to avoid loss of organics. Four mechanical stirrers (FLUCOMATIC 6 system, SELECTA S.A) were used at a speed of 100 rpm to mix the pre-treatment reactors. The concentrations of FNA tested were 0, 2.49, 3.55 and 4.62 mg N-HNO_2/L corresponding to 0, 350, 500 and 650 mg N-NO_2^-/L at pH 5.5 (Table 1). The FNA concentration was calculated using the formula $N\text{-}HNO_2=(S_{N\text{-}NO2})/(Ka*10^{pH})$ with the Ka value found from $e^{-2300/(273+ºC)}$ for a given temperature [18]. A certain volume of a nitrite stock solution (118.3 g $NaNO_2$/L) was added at the beginning of each batch test to achieve the desired nitrite concentration (Table 1). pH was controlled at 5.5 ± 0.1 by using 1.0 M HCl solution.

For each FNA concentration test, sludge samples were withdrawn at different exposure times (2, 5 and 8 hours) to evaluate both the effect of the exposure time and the FNA

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concentration in the WAS characteristics. The pre-treatment assays were carried out at room temperature (~ 25ºC).

2.3. Biochemical Methane Potential (BMP) tests

BMP tests were used to quantify methane production from FNA pre-treated and non pre-treated sludge. The BMP tests were conducted in 250 mL serum bottles (with a working volume of 100 mL). BMPs were set up with an inoculum to pre-treated WAS ratio of 2.4 on a dry VS basis. Each BMP test contained 80 mg of inoculum and 20 mg of WAS pretreated with FNA (0, 2.49, 3.55 and 4.62 mg N-HNO_2/L; see Table 1) according to Zahedi et al. [19]. Ratios inoculum/ Substrate (I/S) between 2 and 4 are required to ensure the proper performance of BMP tests [20,21]. A control test (WAS without nitrite and pH control) was also conducted. The bottles were sealed and stored in a temperature controlled incubator at 37ºC. All the bottles were continuously shaking at 150 rpm to ensure sufficient mixing.

Three sets of blanks (blanks I, II and III) were also conducted. Blank I contained inoculum and Milli-Q water without WAS. Blanks II and III were identical to blank I except with the addition of nitrite stock solution, which resulted in an initial nitrite level of around 70 and 130 mg N-NO_2^-/L, respectively, in blanks II and III. This was done to evaluate the effect of nitrite on the performance of the inoculum. The initial nitrite levels of 70 and 130 mg N-NO_2^-/L in blanks II and III were similar to the lowest and the highest initial nitrite levels found in the BMP tests conducted with pre-treated WAS.

All tests were done in triplicates. The BMP tests lasted for 40 days, when no biogas production was detected. The biogas production was monitored on a daily basis during the first 10 days and every 2-4 days afterwards. The biogas production from the WAS was obtained by subtracting the biogas production from the inoculum (Blank I). MP (milliliters of methane produced) and SMP (milliliters of methane produced per gram of

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VS added) have been expressed under normal pressure (P=1 atm) and temperature conditions (Tª= 0ºC).

2.4. Analytical methods

TS, VS, soluble Kjeldahl nitrogen (SKN) and SCOD were determined according to standard methods [22] and Zahedi et al. [23,24]. NH_4^+ was analyzed via ion chromatography (ICS5000, DIONEX). Biopolymers (proteins and carbohydrates) were measured in the soluble phase before and after pretreatment. To separate solid particles from soluble phase, sludge was centrifuged during 10 min at 13000 rpm and the supernatant was filtered through a 0.20 µm pore size glass fiber filter. Proteins were measured with the Folin Phenol Reagent according to Lowry [25] and Peterson [26] and carbohydrates were measured using a colorimetric method (fenol plus sulfhidric acid) according to Dubois [27].

The biogas volume was measured with a pressure sensor PM7097 (IFM electronic) at the start of each sampling event at the headspace of the BMP bottles. Cumulative gas production was calculated from the pressure increase in the headspace volume (150 mL). CH_4 concentration in the biogas was measured using an infrared specific CH_4 sensor: GasTech S-Guard (GIR-3000 Model). This sensor was calibrated using a commercial 100% CH_4 bottle (Abelló Linde S.A.).

The Live/Dead® BacLight™ bacterial viability kit (Molecule Probes, L-7012) was used to discriminate between viable and dead cells [28] . The BacLight™ bacterial viability kit contains green-fluorescent nucleic acid stain SYTO® 9 and red-fluorescent nucleic acid stain Propidium Iodide (PI). When used alone, the SYTO® 9 stain generally labels all bacteria with intact or damaged membranes. In contrast, PI stain only penetrates bacteria with damaged membranes, causing a reduction in the SYTO® 9 stain fluorescence when both dyes are present. For this reason, bacteria with intact cell

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membranes (assumed to be viable cells) are stained green, whereas bacteria with damaged membranes (dead cells) are stained red fluorescence. Sludge samples (1 mL) were transferred into 10 mL falcon tubes together with 9 mL of Milli-Q water. 1 mL of diluted sludge was transferred into a 2 mL Eppendorf tube in conjunction with 3 µL of a SYTO® 9 and PI mixture solution, and incubated in the dark for 15 min at room temperature, allowing the staining reaction to complete. Then, slides with stained sludge samples (20 µL on each slide) were prepared and observed under a Nikon CS1 confocal laser-scanning microscope (CLSM) using Plan-Apochromat 63xoil objective. The area of the green fluorescence (viable cells) was quantified as a percentage of the total fluorescence area (red + green fluorescence) within each image using a pixel counting program. 20 images were taken from each sample to determine the mean percentage.

2.5. Statistical Analysis

To determine the significance of differences in the parameters studied, factor Analysis of Variance ANOVA was used with significance levels of 0.05. To identify the most important parameter(s), FNA concentration or pre-treatment time, from the solubilization and methane yield results linear relationships were considered. Data analysis and linear relationships were determined by Microsoft Excel software (2010). Graph processing were completed by SigmaPlot 11.0.

3. Results and discussion

3.1 Biocidal effect of FNA on WAS.

Viability was the parameter chosen to demonstrate the toxicity of the FNA on WAS. Fig. 1 shows the percentages of viable microorganisms after being exposed during 2, 5 and 8h to different FNA concentrations. A biocidal effect of FNA on microbial cells is clearly observed in all cases.

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The WAS contained around 90% of viable cells and its viability was hardly affected despite of its exposure to pH 5.5 up to 8 h. When FNA was added, sludge viability substantially decreased. The main difference among the tests where FNA was added was detected after a 2 h of exposure time ($P < 0.05$), where the lowest FNA concentration resulted in a 50% decrease in viable cells while the viability on the highest FNA concentration tested decreased to 20%. At higher exposure times, the viability of the WAS was reduced to very low levels, independently of the concentration of the FNA added. Similar results (no significant differences ($P > 0.05$) in the damaged cell at different FNA concentrations (between 0.1 and 0.3 mg $N\text{-}HNO_2/L$) were observed by Jiang et al. [29]. They used FNA as a biocidal agent in anaerobic sewer biofilms and also obtained levels of 85-95% of damaged cells after exposing the biofilm to 0.1 mg $N\text{-}HNO_2/L$.

3.2. Effect of FNA pre-treatment on fermentation and organic matter reduction

The influence of the FNA concentration and PT on the different organic and nitrogenous compounds of the WAS is shown in Figure 2.

A slight increase of ammonia, SCOD, proteins, carbohydrates and SKN was detected in the WAS maintained at pH 5.5 but without FNA (0 mg $N\text{-}HNO_2/L$), suggesting some solubilization of this sludge even without FNA. When FNA was added, independently of the concentration used, SKN, SCOD, proteins and carbohydrates increased to higher levels as compared with the tests without FNA.

Figure 2A shows that an increased FNA level and exposure time resulted in an increased of SCOD ($P < 0.05$). The highest increase in SCOD (141±0 mg COD/g VS) was obtained at the highest FNA level tested (4.62 mg N/L) and the highest exposure time (8 h), indicating that WAS subject to FNA pre-treatment under these conditions

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was solubilized 54 % more than without FNA pre-treatment (0 mg N/L) and by 170% more than without FNA pre-treatment and without pH control.

Analogously, the trends of soluble proteins and polysaccharides that are the primary compounds of intracellular constitution and exopolymeric substances were similar with the ones found for SCOD (Fig. 2B, 2C), increasing with longer PTs regardless of the FNA concentration and with higher FNA concentration regardless of the PTs. The soluble proteins and polysaccharides increased from 3.7 mg/g VS and 2.7 mg/g VS (exposing to 0 mg N-HNO_2/L and without pH control) to 15.8 mg/g VS and 85.5 mg/g VS when WAS was pre-treated at 4.62 mg N-HNO_2 /L for 8 h, respectively. The decrease in specific ammonia concentration could be attributed to the inhibitory/toxic effect of FNA on sludge hydrolytic enzymes (e.g. protease) and/or enzymes responsible for acidogenesis [6,7]. But the increased production of NH_4^+/VS at 8 h seems to be more related with the decrease on VS concentration detected at 8 h than the increase in the concentration of ammonia (see Figure S1 in the supplementary information section).

In short, increasing the FNA concentration and the PTs resulted in an increase in the SKN, SCOD, proteins and carbohydrates. Also, it is important to highlight that the increase in SKN was not linked to an increase on ammonia concentration (Fig. 2D, 2E), indicating that the biomass was not able to hydrolyze the organic nitrogen. Wang et al. [6,7] and Ma et al. [11] also obtained similar results but at much higher exposure times (24 h). The reason could be that FNA and its derivatives such as nitric oxide (NO) and nitrous anhydride (N_2O_3) enhance EPS degradation [11,30,31], solubilizing some of the particulate substrates.

At 8 h of PT a reduction in the WAS biomass concentration (VS) was observed under all the FNA concentrations tested. The VS reduction of the WAS pre-treated with 2.49,

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3.55 and 4.62 mg N-HNO_2/L was 5.2%, 5.4% and 9.9%, respectively. This reduction was higher than the one observed in the WAS without FNA pre-treatment (3.3%). Previous studies also found similar values of sludge reduction with FNA pre-treatment [29]. Jiang et al. [29] showed a volatile suspended solids reduction of 15% with FNA and 8% without FNA. Thus, FNA pre-treatment enhances the solubilisation of organic compounds and reduces the solids concentration. A linear relationship was observed between the reduction on the concentration of VS and the increase of SCOD (see Figure S2a in the supplementary information section). The correlation coefficient, R^2, was 0.87, indicating a good fit to experimental data. To identify the most important parameter(s), dosage or pretreatment time, affecting the solubilization the slopes of the linear relationships between the increase of FNA and PTs on the SCOD were evaluated (see figure S3a and S3b in the supplementary information section).The medium values of the slopes were 12.4±2.9 (Figure S3a) and 12.6±1.4 (Figure S3b). This shows that pre-treatment time and FNA concentration are equally successful at increasing the solubility.

3.3. The effect of FNA pretreatment on methane generation

In order to assess if the nitrite present in the FNA pre-treated biomass could be inhibitory for the inoculum sludge used in the BMP tests, three different tests were conducted using only inoculum sludge and adding nitrite to reach two different nitrite concentrations (70 and 130 mg N-NO_2^-/L mimicking the lowest and highest initial nitrite levels expected in the BMP tests were FNA pre-treated sludge was used as substrate). The results obtained are shown in Figure 3. Interestingly, the tests conducted with nitrite resulted in a slightly higher CH_4 production as compared to the test where nitrite was not added, indicating that nitrite did not cause any detrimental effect on the inoculum sludge at the concentrations tested. The test without nitrite addition (blank I)

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was chosen as the blank to calculate the methane production from the pre-treated sludge [6,7].

Figure 4 shows the cumulative SMP in all the tests conducted. All results presented are already corrected subtracting the methane production obtained in blank I.

The effect of pH was also individually assessed by pre-treating the secondary sludge at pH 5.5 but without adding any nitrite (Figure 4). Exposing the sludge at this pH reduced rather than increased SMP indicating that FNA and not pH was the responsible agent for this CH_4 increase. No linear relationships were observed between FNA and SMP at any PTs (see figure S4a in the supplementary information section). In fact, the highest FNA concentration tested (4.62 mg N-HNO_2/L) did not further improve MP and SMP (Figure 5). Also, no significant differences with the different FNA concentrations to pre-treatment times higher than 5h were observed ($P > 0.05$). The high values of the SMP (mL CH_4 / g VS) at 8 h are more related with VS destruction (Figure 2f) than an increase on MP (Figure 5). VS destruction at 8 h resulted in lower amounts of available organic matter for biogas production during the BMP tests. Because of this, an FNA pretreatment time of 5h is recommended for WAS.

No good correlation between SCOD and SMP (Figure S2b in the supplementary information section) was observed (R^2=0.65). This might be due to the fact that the COD mainly contributing to SMP comes from extracellular polymeric substances and intracellular compounds (non-soluble). Previous studies with FNA observed similar results: the highest increase in the SCOD did not imply the highest increase in the SMP [15,19].

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Considering the results obtained the optimal conditions to enhance the MP were obtained at 2.49 and 3.55 N-HNO_2/L (no significant differences between both were found; $P > 0.05$) and pre-treatment times of 5h.

This is the first study to report the FNA effect at low exposure times on WAS characteristics and its BMP potential. Wang [6] found an increase on SMP (from 201 to 255 L CH_4/kg VS), when secondary sludge was subject to FNA treatment (2.13 mg N-HNO_2/L) and concluded that this pre-treatment was suitable for this type of sludge. However, in their study a fixed pre-treatment time of 24h was used for all the tests. The present paper shows a similar increase on SMP (from 200 to 230±13 L CH_4/kg VS), when secondary sludge was pre-treated with 2.49-3.55 mg N-HNO_2/L during 5 h. Our results indicate the suitability of the FNA treatment for secondary sludge, but at exposure times five times lower (5h) and with relatively low FNA concentrations (2.49-3.55 mg N-HNO_2/L). In addition, our findings highlight the importance of taking into account the possible reduction of volatile solids during FNA treatment which can affect the MP and falsely increase SMP. These findings are important from the WWTP managers since shorter pre-treatment times (resulting in smaller pre-treatment tanks) and higher MP instead of SMP are preferred.

The enhancement on MP obtained with FNA pre-treatment is comparable with the lower range obtained in other reported pre-treatments such as ultrasound, high pressure and lysis or chemical treatments (acid or alkali treatments) which have been reported to enhance methane production from 11 % to 150 % [4]. The most effective sludge pre-treatments are the thermal and mechanical (high pressure) treatments, obtaining more than 50% of increase in methane production as compared with the FNA pre-treatment where the increase on SMP is around 20-30%). However, the cost associated with the implementation of these pre-treatments is high. In this sense, FNA pre-treatment can be

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considered as a low-cost pre-treatment due to: (i) its easy implementation (ambient temperature and mild agitation); and (ii) it can be produced in the same WWTP by the nitritation process of the anaerobic digestion liquor. It is therefore recommended to conduct a full economic and environmental evaluation before choosing the sludge pre-treatment to be applied in each case.

4. Conclusions

The main conclusions from this study are:

- WAS pre-treatment with FNA at low pre-treatment times (up to 5 hours) is a suitable method for enhancing both SMP and MP.
- Pre-treatment times higher than 5 h did not improve methane production. This was because a slight reduction of volatile solids was detected.
- The optimal FNA pre-treatment was 2.49-3.55 mg N-HNO_2/L with a pre-treatment time of 5 h. These conditions resulted in an increase of 20% on MP compared with untreated WAS.

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List of figures:

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Figure 1.

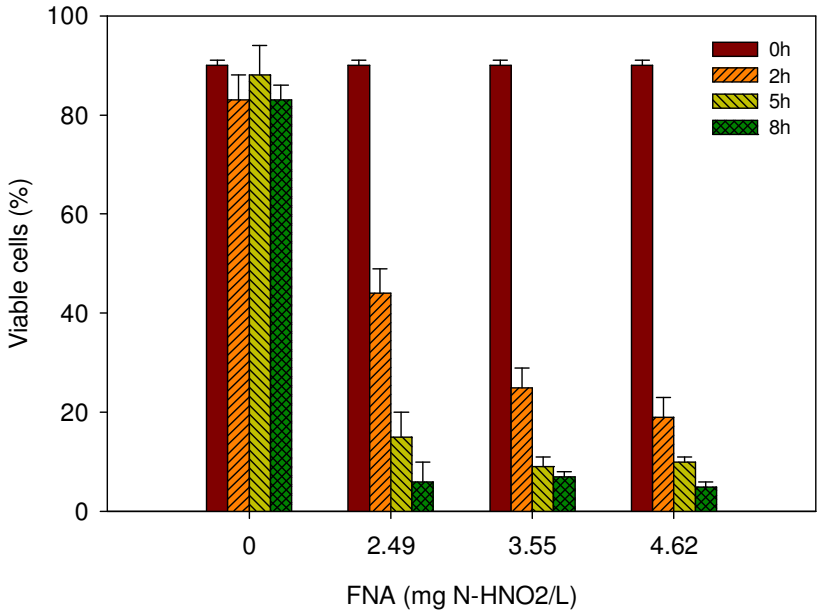

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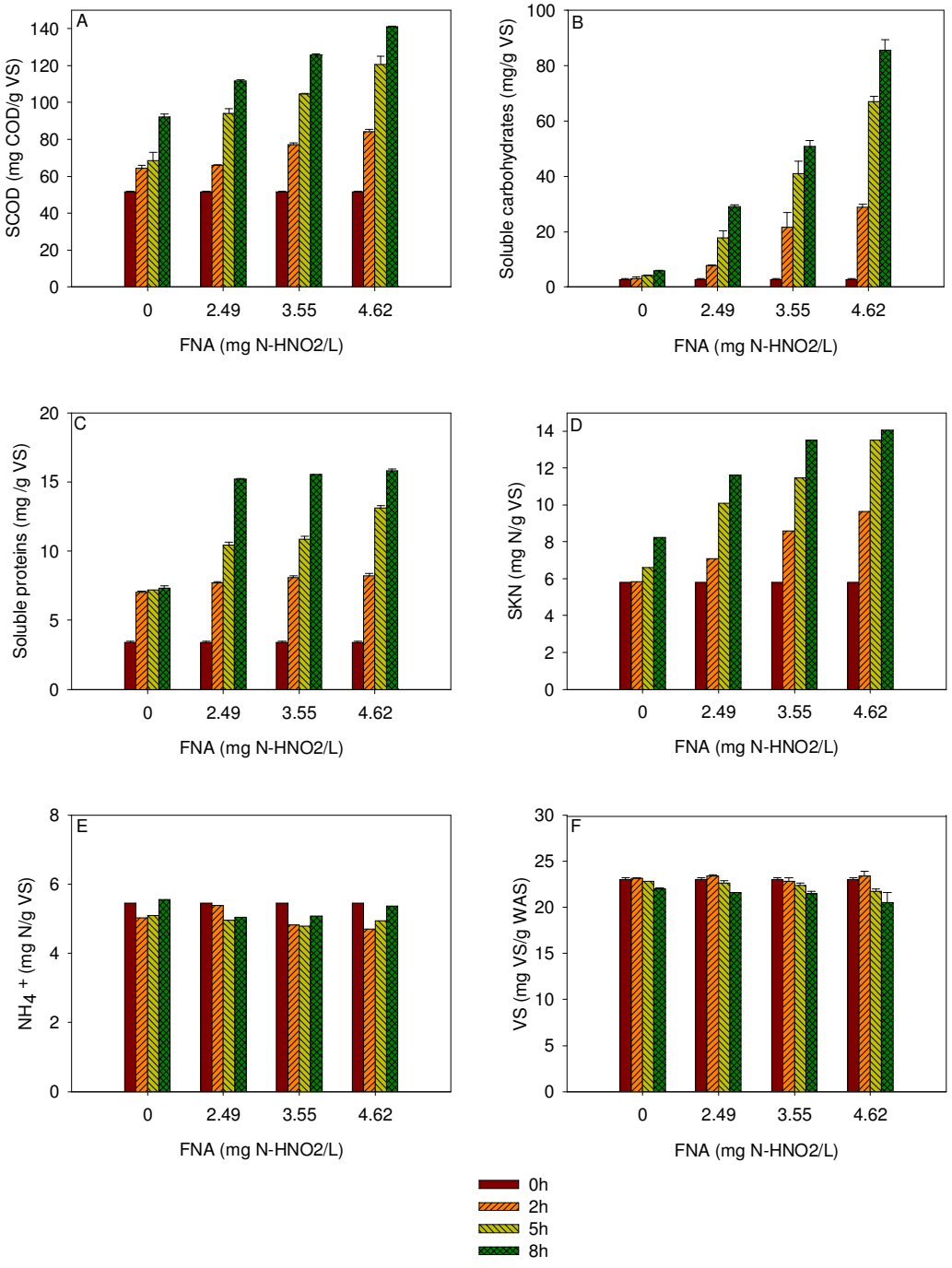
Figure 2.



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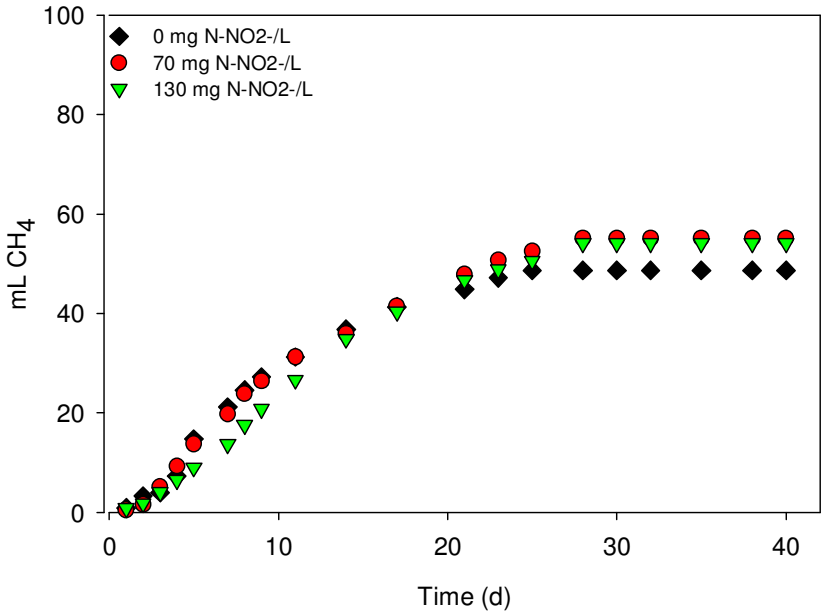

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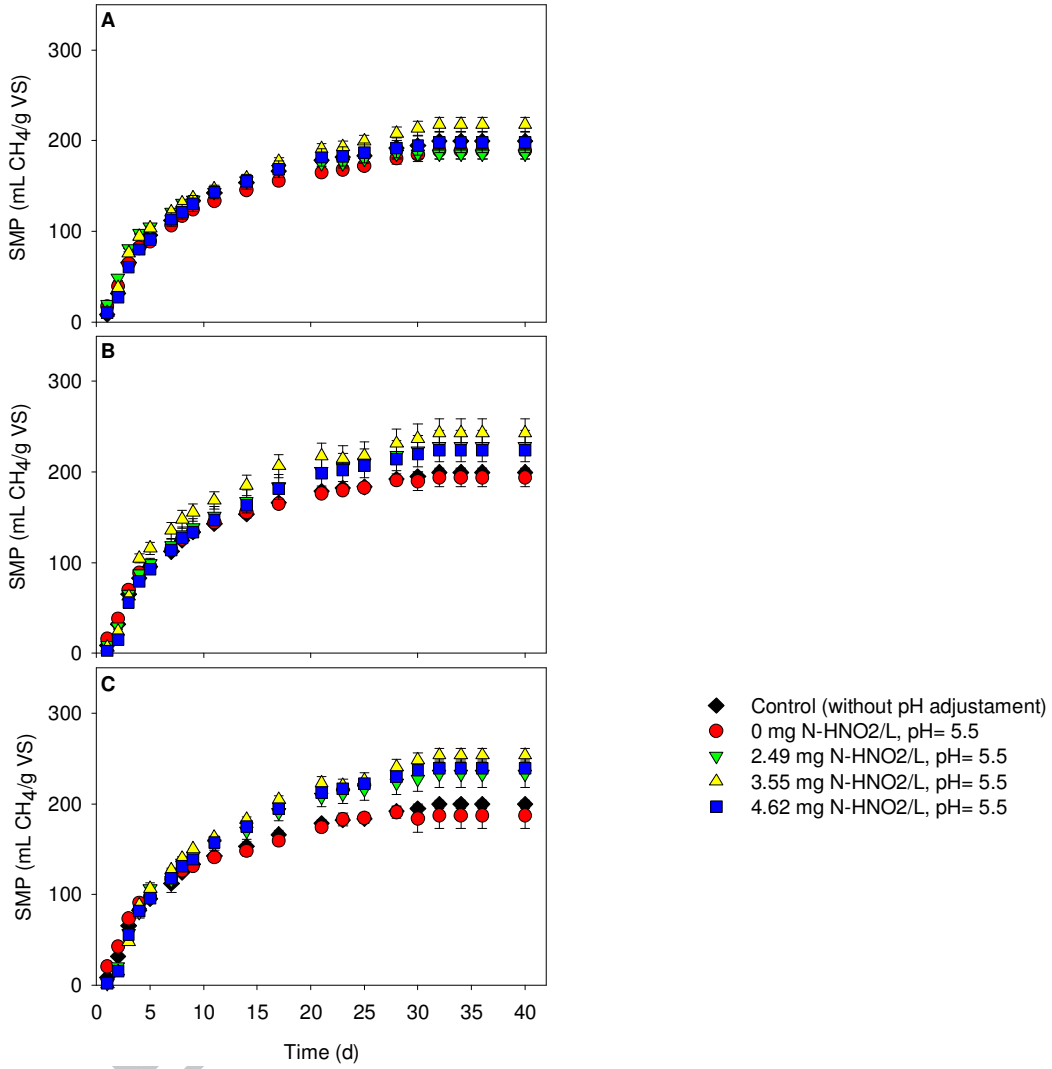

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Figure 5.

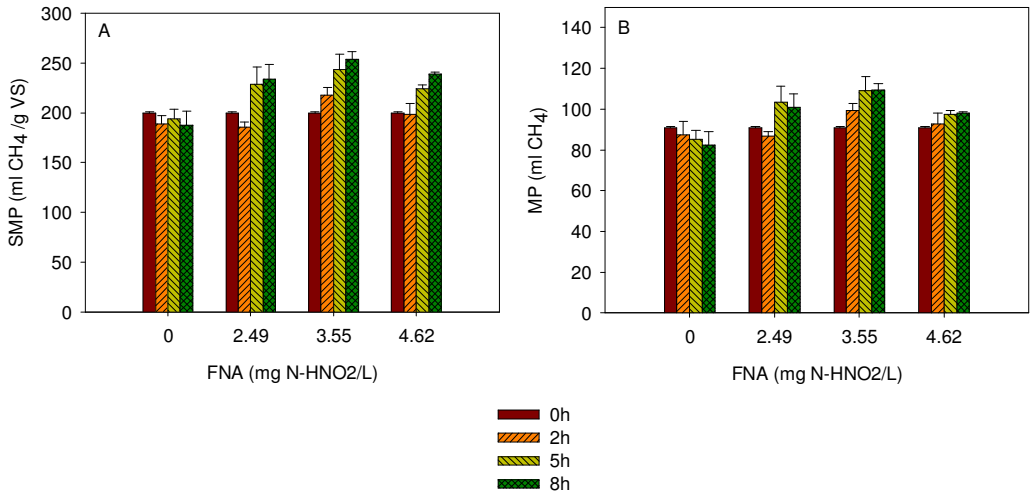

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Table 1. Experimental conditions applied in the pre-treatment, with the FNA concentration varied by adjusting the nitrite concentration and the pH level.

25ºC	**Test1**	**Test 2**	**Test 3**	**Test 4**
FNA (mg N-HNO_2/L)	0	2.49	3.55	4.62
Nitrite (mg N-NO_2^-/L)	0	350	500	650
Nitrite (mg N-NO_2^-/g VS)	0	15.2	21.7	28.3
pH	5.5	5.5	5.5	5.5
Pre-Treatment time (h)				
2	X	X	X	X
5	X	X	X	X
8	X	X	X	X

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Highlights

- Low FNA pre-treatment times (PTs) are effective to improve methane production
- FNA on waste activated sludge (WAS) reduced cell viability to very low levels
- Low FNA pre-treatment PTs on WAS increased the solubility of the organic compounds
- Optimal FNA pre-treatment resulted in an increase of 20 % in methane production

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