



Research Article

Methanogenic toxicity evaluation of chlortetracycline hydrochloride



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ABSTRACT

Background: Anaerobic digestion is a technology applied successfully to converting organic matter into biogas. However, the presence of inhibitory compounds such as antibiotics can adversely affect methane production. The aim of this study is to evaluate the toxic effect of chlortetracycline hydrochloride (CLOR) on the methanogenic bacteria. In order to study the methanogenic toxicity of CLOR, different concentrations of CLOR (10, 50, 100, 200 mg L⁻¹) were evaluated by methanogenic toxicity assays using three feedings.

Results: Maximum methane production was obtained for the assays with 10 mg CLOR L⁻¹, the values obtained were 277 ± 4.07; 193 ± 11.31 and 166 ± 7.07 mL for the first, second and third feedings, respectively. The average values for acetic, propionic and butyric acid at start of the experiments were 2104 ± 139; 632 ± 7.6; 544 ± 26 mg L⁻¹, respectively. The VFA values obtained finally of the experiment were dependent on the evaluated antibiotic concentrations, indicating that the efficiency of methanogenesis is directly affected by the CLOR concentration.

Conclusions: CLOR is an effective methanogenic bacteria inhibitor. Moreover, the results show that CLOR has a bactericidal effect on methanogenic activity given that methane production did not recover during the third feeding. This study shows that the 50% inhibitory concentration (IC₅₀) for methanogenic bacteria in 10 mg L⁻¹.

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1. Introduction

Antibiotics are natural or synthetic chemical substance used extensively in human and animal medicine to treat diseases, prevent infection and promote growth [1,2]. In the environment antibiotics constitute a pollutant, which they enter mainly through discharges from wastewater treatment plants that are not designed to remove them [3,4]. Recent studies report the presence of these drugs in wastewater, groundwater and sewage sludge, with detected concentrations ranging from 0.1 to 100 µg L⁻¹ [5,6,7,8].

Anaerobic digestion (AD) is a technology used to transform organic matter into methane. Organic substrates like sewage, manure, and agricultural wastes may contain antibiotics that inhibit methanogenic activity [9,10,11]. The stability and efficiency of AD processes depend on the microbial population, the biodegradability of the compounds and chemical characteristics [12]. The microorganisms involved in AD are sensitive to antibiotics that can reduce growth rates and disable the activity of microorganisms [10,13,14,15].

In previous works, Álvarez et al. [14] evaluated the effect of oxytetracycline and chlortetracycline hydrochloride (CLOR) during AD of swine manure. They observed that methane production was reduced by 56%, 60% and 62% at oxytetracycline and CLOR concentrations of 10, 50 and 100 L⁻¹, respectively. Arian et al. [15] evaluated the effect of oxytetracycline in calf manure and found a 27% reduction in methane production at a concentration of 3.1 mg oxytetracycline L⁻¹. Furthermore, Sanz et al. [12] observed a reduction in methane production from 20 to 80% when the CLOR concentration increased from 2 to 150 mg L⁻¹.

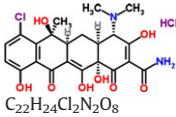
CLOR is a broad-spectrum antibiotic used in human and animal medicine that acts by inhibiting bacterial protein synthesis. Table 1 shows the structure and physicochemical properties of CLOR. The main characteristics are high solubility (156.29 mg L⁻¹) and very low partition coefficient values (Log Kow: -3.60). Consequently, CLOR can inhibit bacteria activity. Sanz et al. [12] showed that CLOR is a powerful inhibitor of anaerobic bacteria, estimating that the 50% inhibitory concentration (IC₅₀) for methanogenic bacteria in 40 mg L⁻¹. They observed that CLOR concentration directly affects the activity of acetogenic and acetoclastic methanogenic bacteria. At concentrations above 200 mg CLOR L⁻¹, bacteria did not consume acetic acid and acetogenic bacteria used to consume butyric acid died at concentrations over 100 mg L⁻¹. They concluded that CLOR has selective effects on different microorganisms.

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Table 1
Structure and physicochemical properties of CLOR.

Analyte (CAS number)	Structure	Molecular weight (g/mol)	Log K_{ow}	Log K_{oc}	Solubility (mg/L)
Chlortetracycline hydrochloride (64-72-2)	 <chem>C22H24Cl2N2O8</chem>	515.15	−3.60	−1.56	156.29

Log K_{ow} y Log K_{oc} was obtained from EPI suite Program version 4.1.

The aim of the present study is to evaluate the toxic effect of CLOR on methanogenic bacteria.

2. Materials and methods

2.1. Analytes

Chlortetracycline hydrochloride (CLOR, 99%) was obtained from Sigma-Aldrich (Steinem, Germany). The volatile fatty acids (VFA; acetic acid, propionic acid and butyric acid), $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, NaOH, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, $\text{Na}_2\text{S} \times 9\text{H}_2\text{O}$, K_2HPO_4 were obtained from Merck (Darmstadt, Germany). NaHCO_3 and NH_4Cl were purchased from Winkler (Santiago, Chile).

2.2. Inoculum

The anaerobic biomass used in the methanogenic toxicity assay was obtained from an anaerobic treatment system of a brewery. This biomass is a granular sludge type. The sludge in the study presented the following characteristics: pH 7.13, volatile suspended solid (VSS) 30.88 mg L^{-1} and total suspended solids (TSS) 48.57 mg L^{-1} . The initial methanogenic activity of the sludge was $0.31 \text{ g COD}_{\text{CH}_4} \text{ g VSS}^{-1} \text{ d}^{-1}$ (COD: chemical oxygen demand).

2.3. Methanogenic toxicity assays

The methanogenic toxicity assays were carried out in 100 mL of total volume (the glass serum bottle volume was 125 mL) using a VFA mixture as substrate and CLOR as the toxic to evaluate, following the methodology previously described [16,17].

Methane production was measured by displacement of an alkaline solution of NaOH 2.5%. The final concentration of each VFA in the reactor (bottle) was: acetic acid 2 g L^{-1} , propionic acid 0.5 g L^{-1} and n-butyric acid 0.5 g L^{-1} (total COD from VFA was $3.8 \text{ g COD-VFA L}^{-1}$). The VFA solution was previously neutralized (pH: 7) with NaOH. The media also contained the following nutrients: NH_4Cl (0.14 g L^{-1}), K_2HPO_4 (0.125 g L^{-1}), $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ (0.10 g L^{-1}), $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ (0.01 g L^{-1}) and NaHCO_3 (0.2 g L^{-1}). The inoculum concentration added to each reactor was $1.77 \text{ g SSV L}^{-1}$. The anaerobic conditions were secured by adding $100 \text{ mg Na}_2\text{S} \times 9\text{H}_2\text{O L}^{-1}$. Each reactor was sealed and bubbled with nitrogen gas for 2 min in order to remove air from the headspace. Finally, samples were incubated at 35°C throughout the experiment.

Three successive feedings to each antibiotic concentration were carried out. In the first feeding, the sludge was exposed to media containing CLOR and VFA substrate. At the end of the first feeding, the spent medium (liquid phase) was carefully decanted and the sludge was again exposed to CLOR and VFA substrate. At the end of the second feeding, the spent medium was removed. In order to evaluate residual sludge activity after the first and second exposure, a third feeding containing only the VFA mixture solution as substrate was added to culture bottles. The assays were carried out at 37°C and incubated for 18 d.

The liquid fraction (supernatant) obtained for each reactor after the feeding was stored and subsequently analyzed (pH, conductivity, redox potential and COD).

The CLOR concentrations evaluated were: 0 (control), 10, 50, 100 and 200 mg L^{-1} . All assays were conducted in triplicate.

2.4. Analytical methodology

Physicochemical parameters: conductivity, redox potential, and pH were measured using a multiparametric OAKTON-PC650 (Eutech Instruments, Singapore).

Water quality parameters: COD, SST and VSS were determined according to the methodologies established in Standard Methods, specifically through the following procedures: the 5220-C method for COD; the 2540-D method for TSS and the 2540-E method for VSS [18].

VFA was determined by gas chromatography (GC) (Shimadzu GC-2014, Kyoto, Japan) equipped with an autosampler (Shimadzu AOC 20i, Kyoto, Japan) and a flame ionization detector (FID), fitted with a $30 \text{ m} \times 0.32 \text{ mm I.D.} \times 0.25 \mu\text{m}$ thickness film Stabilwax-DA column (Restek Corporation - Bellefonte PA, United States). The carrier gas was nitrogen (purity 99.999%) at a constant flow of 2.23 mL min^{-1} . The oven temperature was held at 95°C for 1 min, then temperature programmed at $10^\circ\text{C min}^{-1}$ until 140°C , and finally held for 5 min.

A volume of $1 \mu\text{L}$ of sample was injected in the split mode at an injector temperature of 270°C . The FID temperature was 250°C . The chromatograms obtained were analyzed by GC Solution software, version 2.41 00SU1 (Shimadzu - Kyoto, Japan).

3. Results and discussion

3.1. Effluent characteristics

Table 2 shows the average values and standard deviation ($N = 3$) obtained for the physicochemical parameters (pH, conductivity, redox potential and total COD) from each assay, in function of the evaluated antibiotic concentration.

The presence of CLOR in the reactor did not affect pH during the digestion process. The pH level at the start of the assay was approximately 7 for the blank and each CLOR concentration evaluated, while by the end of the digestion of period (D 18) pH had increased slightly, with values ranging from 7.3 to 8.1. Several authors have reported that the optimum pH range for methanogenic bacteria is between 6.7 and 7.4, while the methanogenesis rate decreases at pH values < 6.2 or > 7.8 [19,20,21]. No negative effects on the microorganisms responsible for AD were observed as a result of any change in pH.

Specific conductivity values ranged from 2.83 to 9.14 mS cm^{-1} . In the absence of CLOR or at low concentrations of the antibiotic (10 and $50 \text{ mg CLOR L}^{-1}$) low conductivity values were observed, while high CLOR concentrations (100 – $200 \text{ mg CLOR L}^{-1}$) were associated with high conductivity values of conductivity, suggesting a relationship between CLOR concentration and conductivity.

Table 2
Characterization of the supernatants obtained for each feeding at the end of the assays.

Parameter/antibiotic (mg L ⁻¹)	First feeding				Second feeding				Third feeding								
	0	10	50	200	0	10	50	100	100	100	200	0	10	50	100	200	
	pH	7.4 ± 0.3	7.9 ± 0.3	7.3 ± 0.8	7.4 ± 0.8	7.9 ± 0.1	7.9 ± 0.7	7.6 ± 0.1	7.6 ± 0.9	7.8 ± 0.1	7.8 ± 0.1	7.8 ± 0.2	7.1 ± 0.5	7.3 ± 0.2	7.8 ± 0.2	7.8 ± 0.2	7.9 ± 0.1
Conductivity (mS cm ⁻¹)	2.8 ± 0.6	4.8 ± 1.4	3.7 ± 1.0	4.1 ± 0.9	4.0 ± 0.4	5.3 ± 0.7	4.2 ± 0.01	4.2 ± 0.01	5.3 ± 0.1	4.3 ± 0.1	4.3 ± 1.3	4.3 ± 0.5	5.5 ± 0.7	7.8 ± 0.3	8.1 ± 0.8	8.13 ± 0.5	8.13 ± 0.5
Redox potential (mV)	-80 ± 5.2	-97 ± 0.1	-106 ± 6.0	-154 ± 17	-90 ± 14	-93 ± 6.2	-102 ± 7.0	-102 ± 7.0	-124 ± 3.7	-105 ± 3.2	-96 ± 9.4	-96 ± 9.4	-82 ± 2.6	-101 ± 4.9	-184 ± 5.3	-190 ± 2.9	-190 ± 2.9
COD soluble (mg L ⁻¹)	359 ± 34	374 ± 5.8	441 ± 8.4	464 ± 14	330 ± 4.3	354 ± 17	403 ± 40	403 ± 40	444 ± 35	334 ± 29	376 ± 29	362 ± 6.6	394 ± 1.2	383 ± 5.9	421 ± 27	421 ± 27	421 ± 27

Anaerobic conditions were evaluated by determining redox potential. For all the analyzed samples the values ranged between -80.4 and -154 mV, indicating that the medium presented anaerobic conditions. COD values were in the range of 253–464 mg L⁻¹ for all samples evaluated in the study. In general, COD decreased by 40% from the concentration measured at the beginning of the assay.

3.2. Methanogenic toxicity

Fig. 1 shows daily methane production for each CLOR concentration evaluated in the different feedings. Maximum methane production was obtained for the assays with 10 mg CLOR L⁻¹ (Fig. 1a), with output levels of 277 ± 4.07; 193 ± 11.31 and 166 ± 7.07 mL for the first, second and third feedings, respectively. Minimum methane production was achieved in assays with a CLOR concentration of 200 mg L⁻¹ (Fig. 1d), with output levels of 210 ± 28.83; 167 ± 33.62 and 128 ± 7.78 mL for the first, second and third feedings, respectively.

Fig. 1 shows that during the first four d of incubation, methane production was lower than 30 mL for all evaluated concentration, independent of the feeding. The low production could have been a result of the microorganisms acclimatizing to the pharmaceutical product [22]. In contrast, maximum methane production was between D 5 and 13, with values ranging from 20 to 265 mL. Finally, in the last stage of the experiment between D 14 and 18 (last stage of the experiment), methane production stabilized as a result of the low activity of the microorganisms. The volume of methane produced ranged between 127 and 277 mL. Shi et al. [23] evaluated the effect of tetracycline and sulfamethoxydiazine on microorganisms responsible for biogas production. They found no presence of methane during the first four days of the assay for any antibiotic concentrations evaluated. However, maximum methane production was observed from D 8 to 12 (D 12: 322 mL d⁻¹). Studying the effect of thiamphenicol, amoxicillin and oxytetracycline on microorganisms, Lallai et al. [24] observed a gradual increase in methane production during the assay period for all the evaluated antibiotics.

Specific methanogenic activity was estimated from daily methane production. Fig. 2 shows the values of methanogenic activity in relation to CLOR concentrations evaluated in the three feedings. Moreover, Fig. 2 shows that the maximum activity was obtained at the first feeding, the estimated values being: 0.5; 0.39; 0.43 and 0.33 mg COD gVSS⁻¹ d⁻¹ for 10, 50, 100 and 200 mg CLOR L⁻¹, respectively.

Methanogenic activity in the second (0.29; 0.26; 0.30 and 0.19 mg COD gVSS⁻¹ d⁻¹) and third feeding (0.10; 0.13; 0.08 and 0.06 mg COD gVSS⁻¹ d⁻¹), with CLOR concentrations of 10, 50, 100 y 200 mg L⁻¹, respectively, were at least 50% lower than after the first feeding.

Table 3 shows the inhibition percentages for each CLOR concentration. The results indicate that the highest percentage of inhibition was obtained for the third feeding, with values of 60.51, 68.79, 75.99 and 81.46% for 10, 50, 100, and 200 mg CLOR L⁻¹. In assessing the inhibition percentage of CLOR in function of concentration, the highest value was recorded for 200 mg CLOR L⁻¹ (44.07, 55.82 and 81.46% for the first, second, and third feeding, respectively).

Stone et al. [25] evaluated the effect of antibiotics tylosin and CLOR in an AD pig slurry treatment and concluded that the presence of CLOR inhibited CH₄ production by 28%. Arikan [26] studied the degradation and metabolization of CLOR by means of AD for at two temperatures, 25 and 55°C. They found that almost 100% of the antibiotic degraded at the higher temperature (55°C); while at the lower temperatures (25°C) the rate of degradation was only 60%. Varel et al. [27] concluded from tests that the percentage of CLOR removed by AD depends on the incubation temperature of the reactor and the assessed antibiotic concentration.

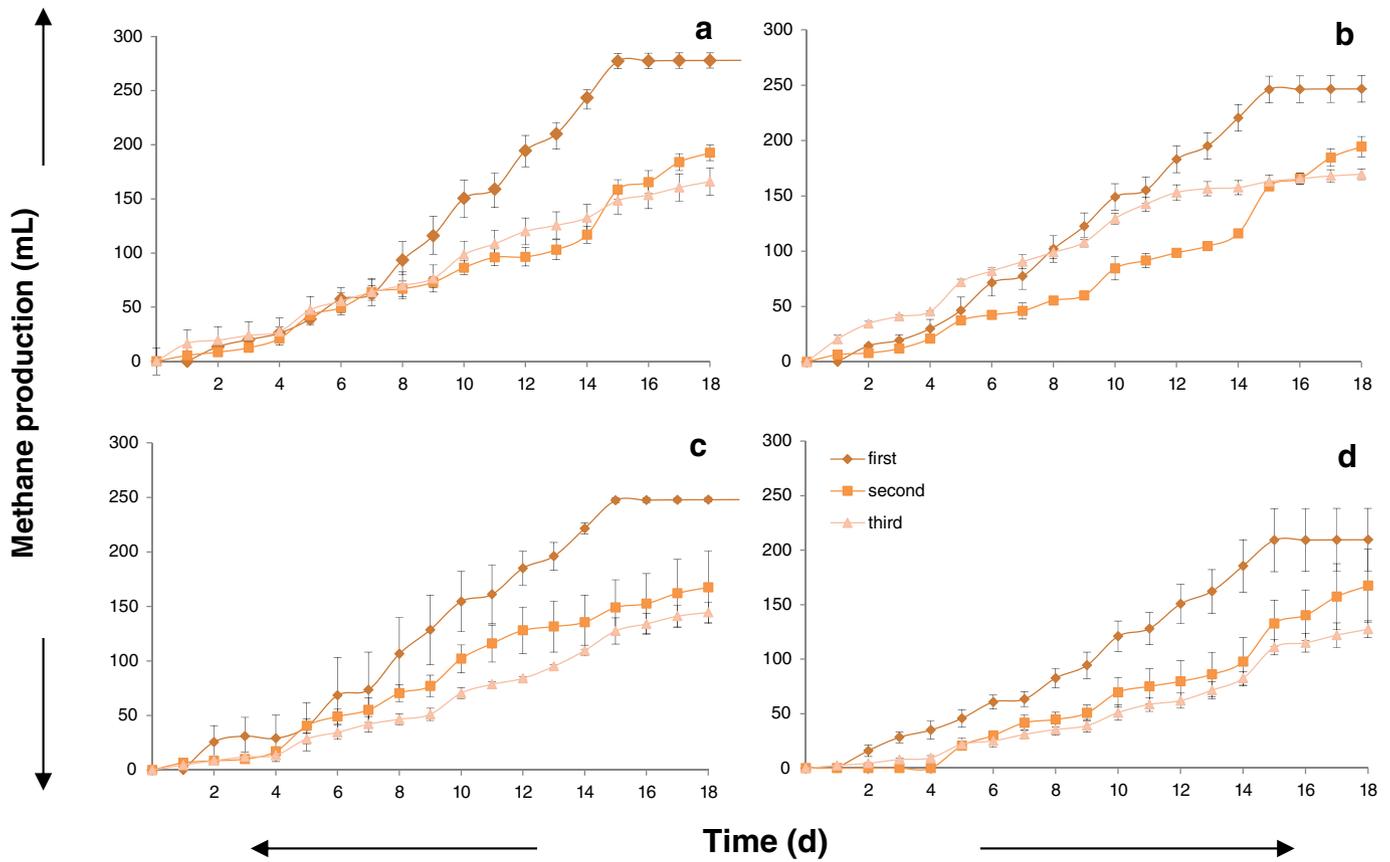


Fig. 1. Methane production (mL) accumulated in the methanogenic toxicity assay of CLOR at different concentration (mg L^{-1}): (a) 10, (b) 50, (c) 100, and (d) 200.

The inhibitory effect of the CLOR increased between the first and second feeding due to continued exposure. Moreover, when the antibiotic was removed in the third feeding, methanogenic activity did not recover. Methanogenic inhibition was evident in all the assays (Table 3). This effect can be explained by the physicochemical properties of CLOR (Table 1). The tetracyclines in CLOR strongly chelate with monovalent and multivalent cations in organic soil matter and inorganic minerals. The adsorption of tetracyclines is strongly governed by their property to ionize as a function of the pH of the medium [28].

The IC_{50} value is defined as the concentration that inhibits 50% inhibition of the measured response of the microbial community. It is a common parameter used to assess inhibitory impact [29,30]. In this study, IC_{50} was calculated in the second feeding at $10 \text{ mg CLOR L}^{-1}$. In this context, Álvarez et al. [14] estimated from the correlation between methanogenic activity and the initial concentration of the antibiotic that $8.9 \text{ mg CLOR L}^{-1}$ reduced methanogenic activity by 50%. Gartiser et al. [31] evaluated the impact of 16 antibiotics on AD and estimated IC_{50} for CLOR at 43.4 mg L^{-1} .

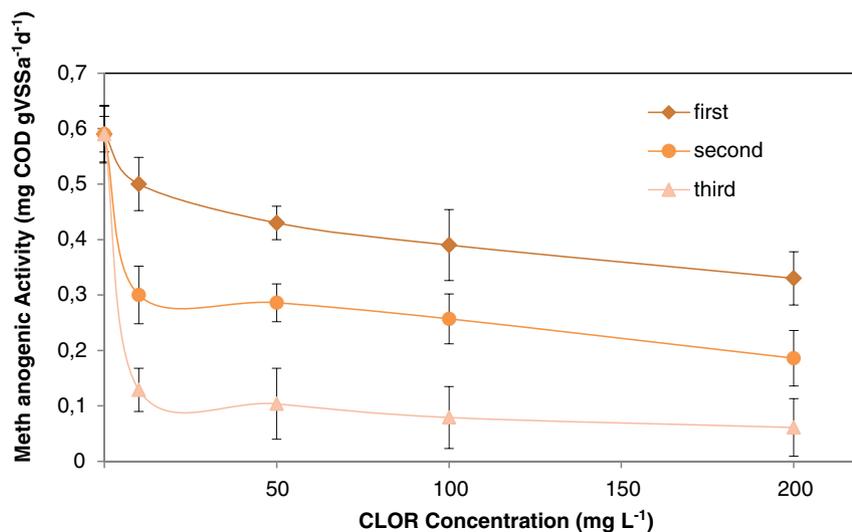


Fig. 2. Methanogenic activity ($\text{mg COD gVSS}^{-1} \text{d}^{-1}$) under different concentrations of CLOR (mg/L) for each feeding.

Table 3
Percentage inhibition (%) of CLOR during the first second and third feeding.

CLOR (mg L ⁻¹)	Feeding (%)		
	First	Second	Third
10	15.3 ± 0.2	22.1 ± 1.8	60.5 ± 1.2
50	27.9 ± 2.5	28.9 ± 0.6	68.8 ± 2.1
100	33.1 ± 0.4	38.7 ± 0.3	76.1 ± 1.4
200	44.1 ± 2.4	55.8 ± 0.9	81.5 ± 0.7

3.3. Volatile fatty acids

Fig. 3 shows the results obtained for the kinetics of VFA transformation versus daily methane production in the second feeding, considering the different evaluated CLOR concentrations.

VFA concentrations were determined on D 1, 4, 7, 9, 12 and 14 to evaluate the efficiency of anaerobic process in the second feeding. The average values for acetic, propionic and butyric acid at start of the experiments (D 1) were 2104 ± 139; 632 ± 7.6; 544 ± 26 mg L⁻¹, respectively. The VFA values obtained at D 14 were dependent on the evaluated antibiotic concentrations, indicating that the efficiency of methanogenesis is directly affected by the CLOR concentration.

Fig. 3a and Fig. 3b show that CLOR concentrations of 10 and 50 mg L⁻¹ did not exhibit an inhibitory effect on the bacteria. Propionic and butyric acid were not detected in the reactor with a CLOR concentration of 10 mg L⁻¹, while the acetic acid concentration was 66.5 ± 9.3 mg L⁻¹.

CLOR concentrations of 100 and 200 mg L⁻¹ (Fig. 3c and Fig. 3d, respectively) were observed to inhibit microorganisms involved in the AD process. The VFA values in the reactor with a CLOR concentration 200 mg L⁻¹ were: 398 ± 38; 69 ± 16 and 14 ± 2 mg L⁻¹ for acetic,

propionic and butyric acid, respectively. The concentration of acetic acid evidences that the acetoclastic methanogenic bacteria responsible for transforming this acid into methane and carbon dioxide (CO₂) were inhibited. These results agree with those reported by Sanz et al. [12] and Stone et al. [25].

Fig. 4 shows the degree of VFA methanization during the second feeding for each CLOR concentration evaluated. By determining the degree of methanization we can evaluate the biodegradability of the substrate (VFA). Fig. 4 shows that on D 1 the average VFA concentration was 4000 g COD L⁻¹, while concentrations at D 14 varied according to the evaluated CLOR concentration. At the highest antibiotic concentration in study (200 mg CLOR L⁻¹), VFA concentration in the reactor was 0.5 g COD L⁻¹, because of which anaerobic digestion was only 70%.

4. Conclusions

CLOR is an effective inhibitor of methanogenic activity. Moreover, results show that CLOR produces a bactericidal effect on methanogenic activity, because methane production did not recover during the third feeding. This study estimated that the 50% inhibitory concentration (IC₅₀) for methanogenic bacteria in 10 mg L⁻¹.

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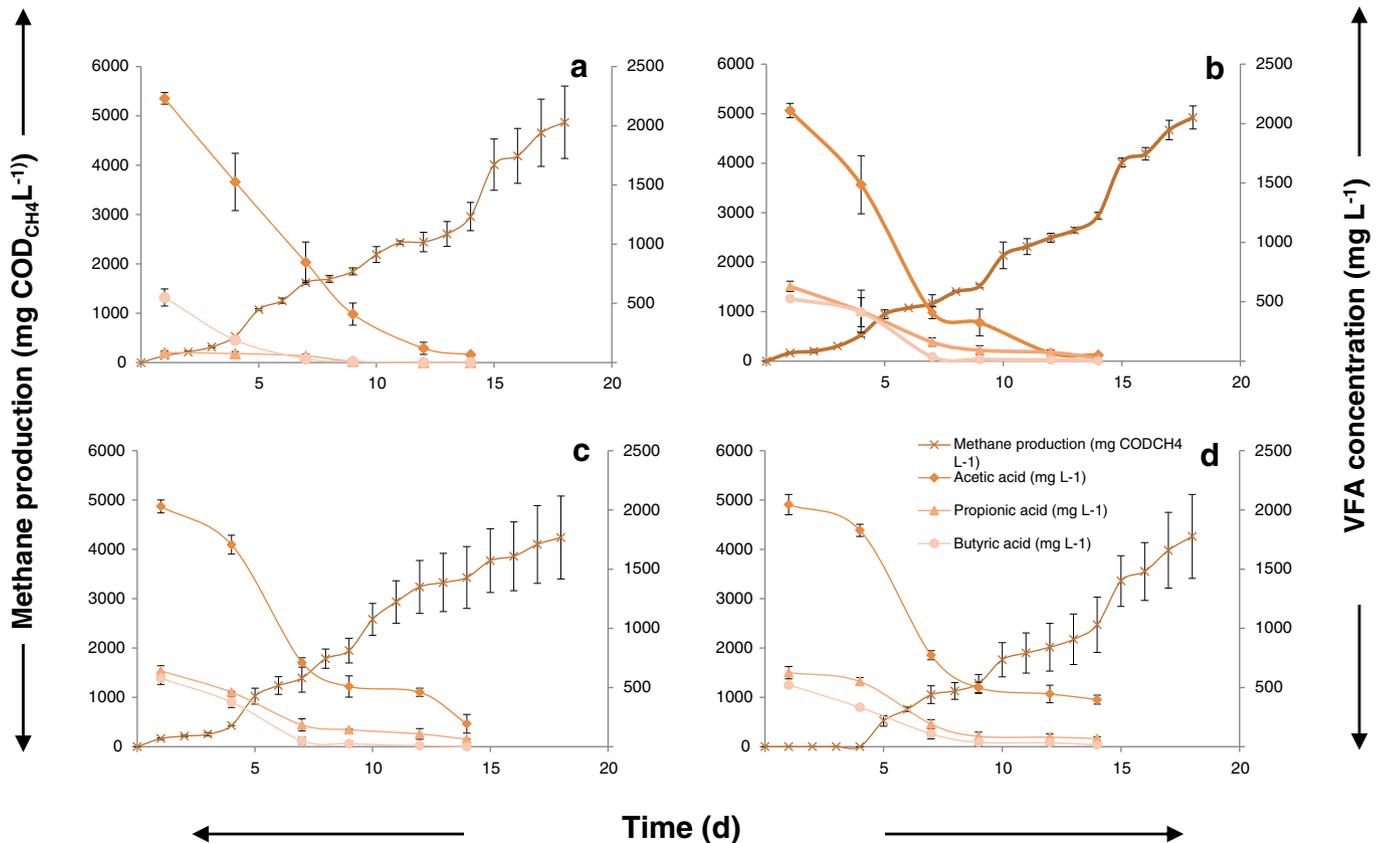


Fig. 3. Cumulative methane production (mg COD_{CH₄} L⁻¹) in methanogenic toxicity assay and the VFA concentration in the supernatant of each reactor evaluated in the second feeding; considering the different concentration of CLOR evaluate (mg/L): (a) 10, (b) 50, (c) 100, and (d) 200.

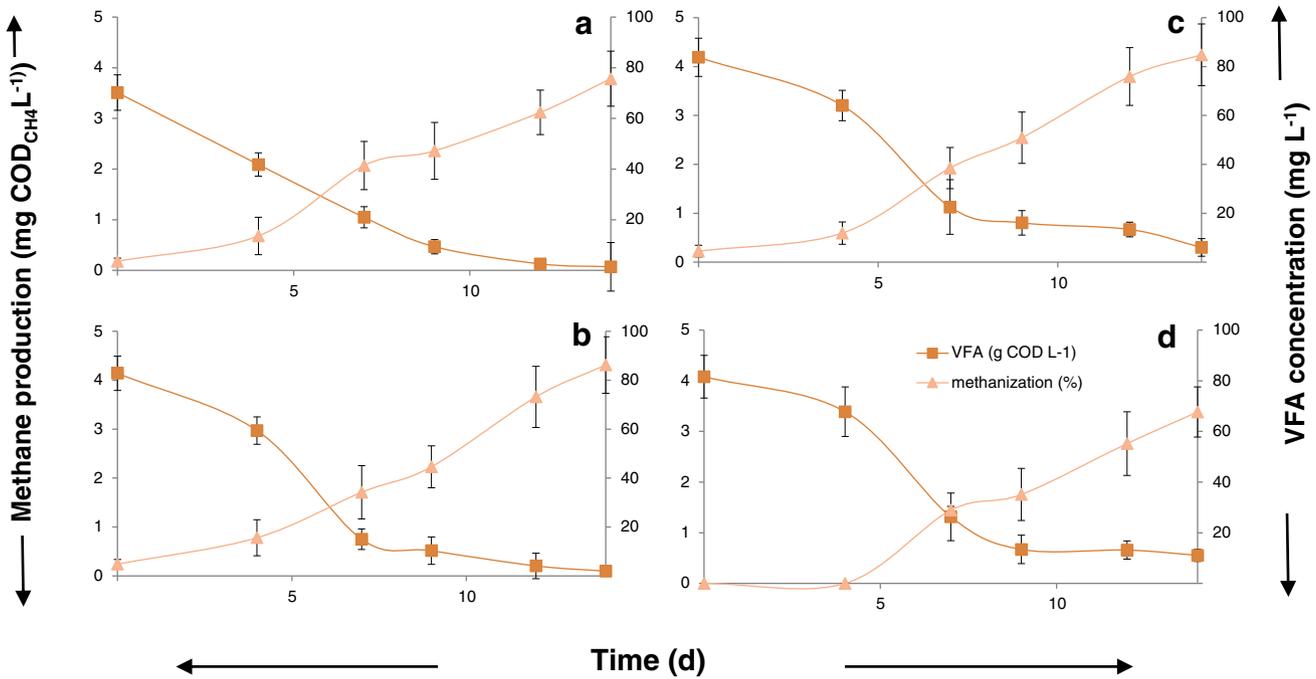


Fig. 4. VFA methanization considering the different CLOR concentrations (mg L⁻¹): (a) 10, (b) 50, (c) 100, and (d) 200.

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