Application of batch tests to assess antibiotic loads in anaerobic processes

Diana C. Rodríguez, Yudy A. Londoño and Gustavo A. Peñuela

ABSTRACT

The presence of antibiotics in drinking water and wastewater has not been widely studied because the sanitary engineering sector mainly focuses on the removal of organic matter and nutrients. There is a lack of environmental regulations for pollutants like antibiotics. Batch tests analyse biodegradability to measure the anaerobic degradation potential of the substrate, or they can be used as toxicity tests. Oxytetracycline, florfenicol (FLO), ceftiofur (CEF) and penicillin G (PEN), commonly used in Colombia for the treatment of livestock diseases, were added in different concentrations to anaerobic sludge contained in serological glass bottles. The production of methane stored in the empty spaces of the bottles was monitored in order to determine the effect of the aforementioned antibiotics on the anaerobic process. It was found that CEF did not have any inhibitory effect on methanogenic activity, while PEN showed inhibition at all concentrations evaluated.

Key words | ceftiofur, florfenicol, inhibition, oxytetracycline, penicillin G

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INTRODUCTION

Antibiotics are one of the most widely used pharmaceutical products in human and veterinary medicine for the treatment of various infectious diseases (Jeong *et al.* 2010). In livestock they are used in subtherapeutic doses as additives to improve the growth of animals without increasing their feeding, and in aquaculture to inhibit fungal growth (Prabhakaran *et al.* 2009). These pharmaceutical compounds are partially metabolized by organisms and are excreted as the same compound or as metabolites before being discharged into wastewater (Prabhakaran *et al.* 2009; Evgenidou *et al.* 2015) or the environment. In the last decade, a large group of pharmaceutical substances have been detected in wastewater treatment plants (Carballa *et al.* 2008; Suarez *et al.* 2008) and antibiotics or their metabolites have been found in manure. These substances can inhibit the anaerobic activity of microbes (Arikan *et al.* 2006, 2008). The presence of antibiotics in ecosystems has been known for 30 years (Vera & Santos 2011). Concern is growing about the adverse effects of these substances on aquatic life and human health (Elmolla & Chaudhuri 2009; Magureanu *et al.* 2015). Several authors have reported that the presence of antibiotics in the environment, even at low concentrations, risks promoting and developing the resistance mechanisms of microbial strains that come into contact with them (Elmolla & Chaudhuri 2009; Prabhakaran *et al.* 2009; Aydin *et al.* 2015).

Penicillin G (PEN) belongs to the class of β-lactam antibiotics (Young *et al.* 2010) and is the main antibiotic used by humans for the treatment of microbial diseases because it inhibits synthesis of the cell wall (Li *et al.* 2008; Young *et al.* 2010). It is widely applied in clinical treatments due to its high anti-microbial activity, broad spectrum, low toxicity, and excellent distribution (Young *et al.* 2010). Florfenicol (FLO) is an antibacterial specially developed for veterinary use. It is used in several countries due to its broad spectrum of activity and effectiveness in controlling various microbial infections in fish (Ferreira *et al.* 2007). Ceftiofur (CEF) is a third generation cephalosporin with a broad spectrum of activity against many organisms commonly cultured from bone infections (Zhang *et al.* 2002). It is part of an important class of antimicrobial drugs used in veterinary medicine (Witte *et al.* 2011) for the treatment of microbial diseases in animals (Rafii *et al.* 2009; Liu *et al.* 2015). CEF is a highly effective drug against almost all Gram-negative and Gram-positive pathogens (Witte *et al.* 2011). Oxytetracycline (OXY) is an antibacterial belonging to the tetracycline group. It has been widely used for decades for the treatment of microbial diseases in aquaculture because of its effectiveness, low cost and high spectrum of activity (Ferreira *et al.* 2007).

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The above compounds may have adverse effects on the biological processes of wastewater treatment. Anaerobic digestion is one of the most frequently used treatment processes as it is a robust technology suitable for treating various types of wastewater and sludge. It has advantages such as the production of biogas as an energy source and a low generation of sludge (Fountoulakis *et al.* 2008). However, the presence of antibiotics in these processes may act negatively on mixed populations of anaerobic microbes, determining which microbial cultures are selected or reducing their growth rate (Lallai *et al.* 2002). This is a condition that significantly influences both the degradation efficiency of the organic load of the waste and the production of biogas (Lallai *et al.* 2002).

The aim of this study was to determine the effect of the antibiotics OXY, FLO, CEF and PEN, which are commonly used to treat cattle diseases in Antioquia, Colombia, on the anaerobic process. These antibiotics were added in various concentrations to anaerobic sludge contained in serological glass bottles, and the production of methane stored in the empty space of the bottles was monitored using gas chromatography.

MATERIALS AND METHODS

The methodologies established by Poirrier (2005), Alvarez *et al.* (2010), Soto *et al.* (1993) and Molina *et al.* (2008) were employed for the tests. All batch tests were performed in triplicate using 60 mL serological glass bottles and a working volume of 20 mL under continuous stirring (100 rpm) at 35 °C.

Preparation of the culture medium

A culture medium was prepared with 50 mL of macronutrients, 10 mL of micronutrients, 0.3 g of yeast extract and 1 mL of 0.1% resazurin. Macronutrients were prepared with 11.4 g/L NH_4Cl, 8.3 g/L KH_2PO_4, 0.16 g/L $CaCl_2$ and 1.9 g/L $MgCl_2$. Micronutrients were prepared with 200 mg/L $FeCl_2.4H_2O$, 240 mg/L $CoCl_2.6H_2O$, 60 mg/L $MnCl_2.4H_2O$, 5 mg/L $CuCl_2.2H_2O$, 6 mg/L H_3BO_3, 7 mg/L (NH_4) $6Mo_7O_{24}$, 7 mg/L $Na_2SeO_3.5H_2O$, 10 mg/L $NiCl_2.6H_2O$, 5.5 mg/L $ZnCl_2$, 100 mg/L EDTA and 0.1 mg/L HCl. The culture medium was sparged with N_2 for 2 min and then 0.5 g/L of cysteine and 5 g/L of $NaHCO_3$ were added. It was verified that the pH was in the range of 7.0–7.2. Finally, the medium was diluted to 1 L with distilled water.

Characterization of the inoculum

The inoculum was obtained from the upflow anaerobic sludge blanket (UASB) reactor of a wastewater treatment plant of the Colanta dairy company, located in the municipality of San Pedro, Antioquia (Colombia). The physicochemical characteristics of the inoculum were: 50.97 g total suspended solids (TSS)/L; 45.76 g volatile suspended solids (VSS)/L; a VSS/TSS ratio of 0.90; a sludge volume index (SVI) of 69 mL/g; a sedimentation velocity (Vs) of 44.3 m/h; a density of 38.78 g/L and an average granule diameter of 0.74 mm.

Validation of the inoculum concentration

In the serological glass bottles, a volume of 60 mL inoculum was added, using concentrations of inoculum from 0.5 g VSS/L to 5.0 g VSS/L; additionally, 1.2 mL of Na_2S (20 g/L) was added as a reducing agent to maintain anaerobic conditions. Finally, a calculated volume of culture medium prepared previously was also added. The serological glass bottles were completely sealed with rubber stoppers and aluminium seals and N_2 was sparged for 2 min in each bottle to displace any oxygen present. To balance out the pressure and the temperature, the bottles were left to undergo mechanical stirring for about 12 hours; subsequently the bottles were purged and a volatile fatty acid (VFA) solution was added, previously neutralized with NaOH, using the following proportions: 0.5 g/L of acetic acid, 0.5 g/L of propionic acid and 0.5 g/L of butyric acid to act as a substrate. For controls, the same volumes of inoculum were employed but without addition of VFA solution.

Validation of the substrate concentration

An adequate concentration of inoculum validated in the previous phase (2.5 g VSS/L) was added to 60 mL serological glass bottles. Furthermore, 1.2 mL of Na_2S (20 g/L) was added as a reducing agent to maintain the anaerobic conditions, along with a calculated volume of culture medium previously prepared. The glass bottles were completely sealed with rubber stoppers and aluminium seals, and N_2 was sparged for 2 min in each bottle to displace the oxygen present. To balance out the pressure, the bottles were left to undergo mechanical stirring for about 12 hours before adding an VFA solution previously neutralized with NaOH using the proportions: 0.0 g/L, 0.1 g/L, 0.5 g/L, 1.0 g/L, 1.5 g/L, 2.0 g/L and 2.5 g/L of acetic acid and 0.5 g/L of propionic acid and 0.5 g/L butyric acid. The concentrations of propionic acid and butyric acid remained constant in accordance with

the methodology used by Soto *et al.* (1993). However, the concentration of acetic acid was varied as this is the most important electron donor for methanogenic microbes.

Tests with the antibiotics

Tests were carried out following the same methodology described in sections Characterization of the inoculum and Validation of the inoculum concentration, using concentrations of inoculum and acetic acid validated previously. The antibiotics used were OXY of the tetracycline group, PEN and CEF of the β-lactamase group and FLO from the phenicol group. These were added in accordance with the experimental design presented in Table 1. Control tests were performed in order to evaluate the methanogenic activity of the biomass without antibiotics and antibiotic stability under anaerobic conditions. The tests were: without antibiotics and without substrate (inoculum), without antibiotics (inoculum + substrate) and without substrate (inoculum + antibiotics). All tests were performed in triplicate at 35 °C and under constant stirring (100 rpm).

Laboratory analysis

Physiochemical analysis

Analyses of VSS, chemical oxygen demand (COD) and pH were performed following the protocols established in *Standard Methods* (APHA 2012).

Antibiotics

The antibiotics were analysed with a high efficiency 1,100 to 1,200 liquid chromatograph with a diode array detector (Agilent Technologies). Separation was performed in a 150 × 4.6 mm ultra IBD analytical column, with a 5 micron film thickness (Restek) and a guard column. The analytical column was maintained at 30 °C. The mobile phase was solvent A (acetonitrile: methanol (9: 1) at 0.1% in trichloroacetic acid) and solvent B (HPLC grade water at 0.1% in trichloroacetic acid), with a gradient that started with 80% solvent B and 20% solvent A, then after 15 minutes changed to 20% solvent B and 80% solvent A. At a flow rate of 1.5 mL/min, 100 µL of the sample was injected. The diode array detector was used at 230, 290, 270 and 356 nm. The run time was 15 min.

Methane

The production of methane in each bottle was measured using an Agilent Technologies gas chromatograph (Model 6890) with a flame ionization detector. A 30 m × 0.32 mm internal diameter HP-PLOT Q column was used. The furnace temperature was maintained at 40 °C, the detector temperature was 300 °C and the injector temperature was 250 °C. The carrier gas was helium at a flow of 54 mL/min. Injection was performed in split mode. The retention time of methane during the chromatographic analysis was 2.43 minutes.

Statistical analysis

Using the StatGraphics 16.1.11 program, a multiple range test was performed to find significant differences between the different methods applied. The mean, standard deviation, coefficient of variance and simple regressions were calculated.

RESULTS AND DISCUSSION

Inoculum concentration

Figure 1 shows the results obtained from specific methanogenic activity (SMA) tests using 10 concentrations of inoculum in a range of 0.5 g VSS/L–5.0 g VSS/L, with and without substrate.

According to Figure 1, the methanogenic activity of the sludge increases as the concentration of VSS increases to 2.5 g VSS/L. From this concentration onwards (3.0 g VSS/L–5.0 g VSS/L), the methanogenic activity becomes constant. Such behaviour can be attributed to various causes; the first is that such high concentrations of inoculum can cause an imbalance in the relationship between the food or substrate and the microorganisms due to there being insufficient food for such a large amount of microorganisms. This leads to starvation, and therefore, the anaerobic microbes stop producing methane. Secondly, although the bottles have free space for storing methane, this gap may reach capacity and begin to exert pressure on the sludge. This would stop them from storing more methane and negatively affect the microbes. The concentration of anaerobic sludge required for subsequent tests is 2.5 g VSS/L. This value can be found referenced in the literature, and higher concentrations would imply an unnecessary expense of sludge. Using this sludge concentration, the average MA value was 0.862 g COD-CH_4/g VSS d. According to studies by Harper & Pohland (1986), the maximum methanogenic activity for pure or enriched cultures is about 10 g

Table 1 | Experimental design for the evaluation of antibiotics

N°	Test	Inoculum (g VSS/L)	Acetic acid (g/L)	Propionic acid (g/L)	Butyric acid (g/L)	OXY (mg/L)	FLO (mg/L)	PEN (mg/L)	CEF (mg/L)
1	Inoculum	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	Inoculum + substrate	2.5	1.5	0.5	0.5	0.0	0.0	0.0	0.0
3	Inoculum + OXY	2.5	0.0	0.0	0.0	0.2	0.0	0.0	0.0
4						0.5			
5						1.0			
6						5.0			
7						10.0			
8	Inoculum + substrate + OXY	2.5	1.5	0.5	0.5	0.2	0.0	0.0	0.0
9						0.5			
10						1.0			
11						5.0			
12						10.0			
13	Inoculum + FLO	2.5	0.0	0.0	0.0	0.0	0.2	0.0	0.0
14							0.5		
15							1.0		
16							5.0		
17							10.0		
18	Inoculum + substrate + FLO	2.5	1.5	0.5	0.5	0.0	0.2	0.0	0.0
19							0.5		
20							1.0		
21							5.0		
22							10.0		
23	Inoculum + PEN	2.5	0.0	0.0	0.0	0.0	0.0	0.2	0.0
24								0.5	
25								1.0	
26								5.0	
27								10.0	
28	Inoculum + substrate + PEN	2.5	1.5	0.5	0.5	0.0	0.0	0.2	0.0
29								0.5	
30								1.0	
31								5.0	
32								10.0	
33	Inoculum + CEF	2.5	0.0	0.0	0.0	0.0	0.0	0.0	0.2
34									0.5
35									1.0
36									5.0
37									10.0
38	Inoculum + substrate + CEF	2.5	1.5	0.5	0.5	0.0	0.0	0.0	0.2
39									0.5
40									1.0
41									5.0
42									10.0

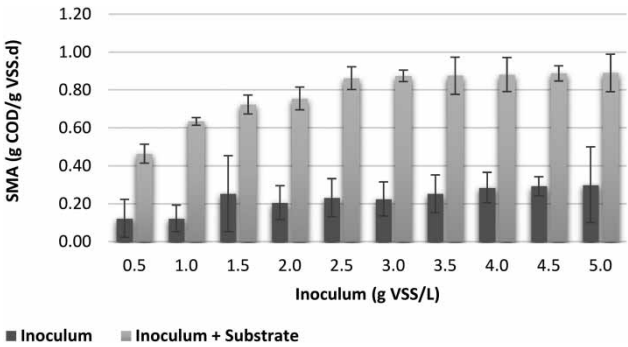

Figure 1 | Inoculum validation test. Error bars indicate deviations of the data.

COD-CH_4/g VSS d. However, Dolfing & Bloemen (1985), Field *et al.* (1988) and Guiot (1991) established that the methanogenic activity of sludge from laboratory and industrial scale digesters is in the range of 0.1 g COD-CH_4/g VSS d–1.0 g COD-CH_4/g VSS d. This is because the latter sludge generally contains between 1% and 10% strictly methanogenic microbes. On the other hand, Poirrier (2005) employed a range of 1–2 g VSS/L in tests of hydrolytic, acidogenic and methanogenic activity. Alvarez *et al.* (2010) used a value of 2 g VSS/L of inoculum, while Chelliapan *et al.* (2006) used a higher value of 5.8 g VSS/L. Taking into account the performance coefficient of *Methanosarcina* spp. and the substrate concentration used, Soto *et al.* (1993) determined by mathematical calculation that the optimal range of inoculum should be between 0.8 and 8.0 g VSS/L in order to generate methanogenic activity of up to 1.0 g COD-CH_4/g VSS d. It can be seen that the reported inoculum concentration varies widely. Therefore, the concentration must be validated by taking into account the sludge type prior to assembly of the tests. Methanogenic activity testing and the selection of a suitable microbial population are essential for the proper operation of anaerobic reactors. According to Hulshoff *et al.* (2004), the capacity of a reactor to retain biomass depends largely on its ability to form aggregates or granules that can reach a methanogenic activity of up to 2 g COD-CH_4/g VSS d.

The control (inoculum without substrate) in Figure 1 verifies that without the presence of the substrate the inoculum has a very low SMA. This tends to be almost constant in every test. The values found are due to the transformation of the organic carbon contained in the microbial cells. Theoretically, the biomass has a composition of $C_5H_7NO_2$. As there is no carbon source, the microbes try to follow a degradation pathway that starts with the organic carbon and ends in cell death. This is reflected in the high variability of the data, which leads to much higher deviations.

Substrate concentration

Figure 2 shows the results obtained using different concentrations of acetic acid and the same amounts of propionic and butyric acids (0.5 g/L for both). As the acetic acid concentration was increased to 1.5 g/L, the methanogenic activity also increased. However, for a concentration of 2.0 g/L, the methanogenic activity remained very constant compared with the previous data. The methanogenic activity for 1.5 g/L was 1.123 g COD-CH_4/g VSS d, and for 2.0 g/L it was 1.131 g COD-CH_4/g VSS d. This means that 1.5 g/L of acetic acid is sufficient to generate good activity in microbes, and a concentration of 2.0 g/L would imply an unnecessary expense of acid. On the other hand, for a higher concentration of acetic acid (2.5 g/L), methanogenic activity decreased to an average value of 0.911 g COD-CH_4/g VSS d, implying that excess substrate leads to the inhibition of methanogenic microorganisms, possibly associated with changes in pH and alkalinity consumption in the system.

According to studies by Wu *et al.* (1991) and Guiot (1991), the values of SMA are related to the type of substrate used. Generally, higher values ranging from 1 to 2.6 g COD-CH_4/g VSS d are found for the formate substrate and the H_2/CO_2 gas mixture used by the hydrogenotrophic methanogenic microorganisms. However, when acetate is used as a substrate, the methanogenic activity values can range from 0.1 to 1.1 g COD-CH_4/g VSS d. In this case, the substrate is used by acetoclastic methanogenic microorganisms. Soto *et al.* (1993), assessed the methanogenic activity for different concentrations of acetic acid. They found that for higher concentrations (2 g/L) the methanogenic activity became

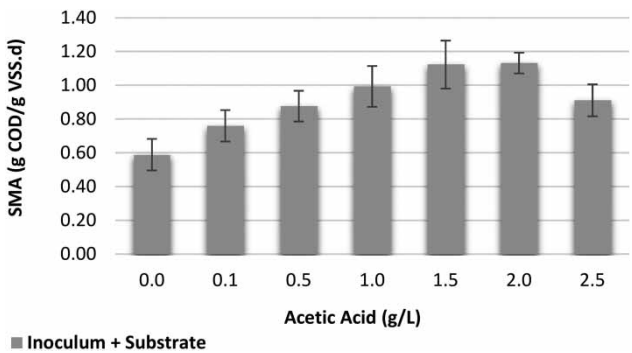

Figure 2 | Substrate validation test. Error bars indicate deviations of the data.

constant, and concentrations of 1 g/L gave a methanogenic activity value of approximately 0.8 g COD-CH_4/g VSS d, which is slightly lower than the value found in this study. Bonastre *et al.* (1987) used a mixture of acetic and propionic acids at a ratio of 1.5 g/L: 0.5 g/L, which is very similar to the ratio employed in this study.

For the inoculum control, which contained propionic and butyric acid but no acetic acid, methanogenic activity was exhibited, but it was low (0.589 g COD-CH_4/g VSS d). This indicates that the main substrate is missing and that methanogenic microbes use acetic acid much better as an electron donor to carry out their transformation into methane.

Tests with antibiotics

From the validated concentrations of inoculum and substrate (VSS 2.5 g/L and 1.5 g/L acetic acid), different concentrations of the four selected antibiotics were added (OXY, FLO, PEN and CEF) to determine the production of methane over time. Figure 3 shows the SMA results using different concentrations of OXY. An OXY concentration of 0 mg/L produces the maximum methanogenic activity (1.192 g COD-CH_4/g VSS d), which corresponds to the inoculum with the substrate (a mixture of acetic acid + propionic acid + butyric acid). However, as the antibiotic is added, the SMA progressively decreases to an average value of 0.702 g COD-CH_4/g VSS d for 10 mg/L of OXY.

When performing the multiple range test to determine which measurements were significantly different from one another, it was found that five pairs had statistically significant differences with a confidence level of 95.0%. These pairs had among the lowest concentrations with respect to the highest, i.e. 0.2–5.0 mg/L, 0.2–10.0 mg/L, 0.5–5.0 mg/L, 0.5–10.0 mg/L and 1.0–10.0 mg/L, indicating that in the case of OXY, concentrations of 0.2 mg/L, 0.5 mg/L and 1.0 mg/L do not present statistically significant differences. This suggests that the microbes are not affected by the presence of this antibiotic at the above concentrations, while for 5.0 mg/L and 10.0 mg/L the decrease in SMA can be statistically verified.

Additionally, control tests were performed with the inoculum and the antibiotic without the addition of substrate (Figure 3), demonstrating that for almost all concentrations employed the SMA production was as low as that when the inoculum was used alone. Therefore, OXY does not represent a source of substrate for these microorganisms. Figure 4 shows the results of SMA against FLO. The SMA average, without the antibiotic, is 1.192 g COD-CH_4/g VSS d. This value was the same for all tests. However, the SMA decreased considerably after the first dose of FLO was applied (0.2 mg/L), reaching a value of 0.403 g COD-CH_4/g VSS d with a FLO concentration of 10 mg/L.

After performing the multiple range test, a statistically significant difference was found between the FLO concentration of 0.2 mg/L and other concentrations (five pairs show statistically significant differences with a confidence level of 95.0%). This indicates that for each concentration inhibition occurs in the microbes, which prevents the adequate production of methane. Figure 4 shows that inhibition begins at 0.2 mg/L, for which the SMA is 0.963 g COD-CH_4/g VSS d. This is lower than the initial value.

Regarding the substrate tests (Figure 4), as expected due to the inadequate carbon source, there was little SMA production, and that which was generated occurred as a result of starvation. However, from 0.5 mg/L to 10 mg/L the SMA is even lower than the control (without the antibiotic), confirming the inhibitory effect of this antibiotic.

Figure 5 shows the variation of SMA when using PEN. It is evident that PEN causes a strong inhibition in the anaerobic microorganisms in all of the applied concentrations. The lowest concentration of PEN (0.2 mg/L) gave an SMA of 0.476 g COD-CH_4/g VSS d and the highest concentration

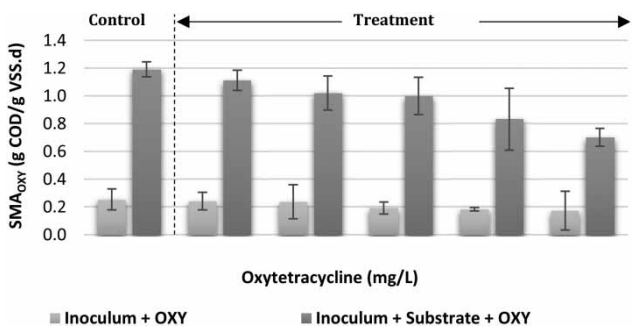

Figure 3 | Effect of OXY on SMA. Error bars indicate deviations of the data.

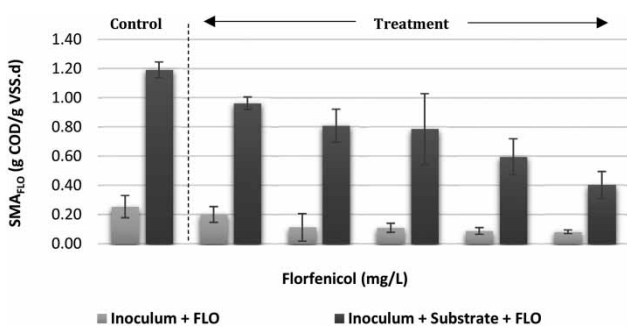

Figure 4 | Effect of FLO on SMA. Error bars indicate deviations of the data.

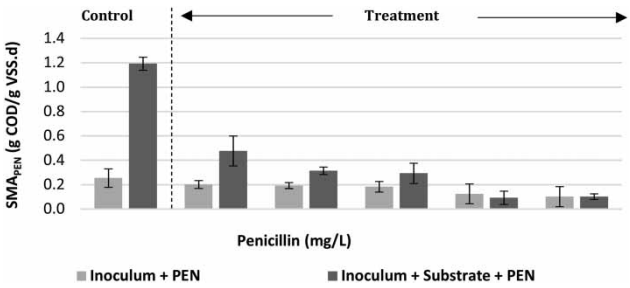

Figure 5 | Effect of PEN on SMA. Error bars indicate deviations of the data.

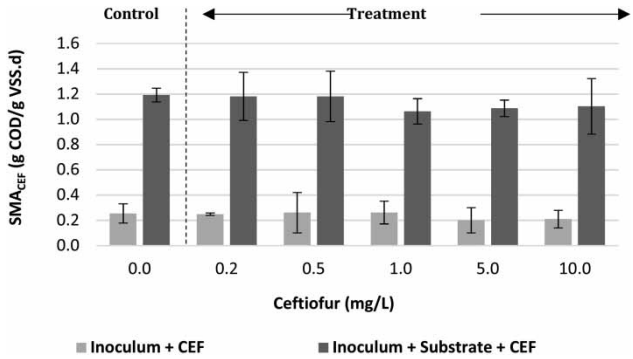

Figure 6 | Effect of CEF on SMA. Error bars indicate deviations of the data.

(10.0 mg/L) gave an SMA of 0.101 g COD-CH_4/g VSS d. In all cases, the initial SMA was 1.192 g COD-CH_4/g VSS d, which corresponds to the inoculum with the substrate and without the antibiotic. Although the same result was seen for FLO, for PEN the decrease in SMA is much higher, suggesting that this antibiotic has a greater effect on anaerobic metabolism.

Using the multiple range test, a statistically significant difference was found between the concentration of 0.2 mg/L of PEN and the other concentrations (six pairs show statistically significant differences with a confidence level of 95.0%). There is also a difference between the concentration of 0.5 mg/L and the two higher concentrations (5.0 mg/L and 10.0 mg/L). Furthermore, it can be seen that between 5.0 mg/L and 10.0 mg/L of PEN no statistical difference was found, which may indicate that after 5.0 mg/L the inhibitory effect is so high that it remains constant. The PEN tests without substrate (Figure 5) follow the same trend as the FLO.

Figure 6 shows the results of SMA for the antibiotic CEF. Unlike the results of the other antibiotics, very high inhibitions were not found for CEF. The SMA data remained very constant. In the case of the highest concentration (10.0 mg/L of CEF), the SMA average was 1.102 g COD-CH_4/g VSS d, which was very close to the initial value (without antibiotics) of 1.192 g COD-CH_4/g VSS d.

The multiple range test shows that there is no statistically significant difference between the comparisons made (0 pairs show statistically significant differences with a confidence level of 95.0%). Thus, for the concentrations of CEF tested, it can be concluded that the microbes do not suffer any inhibition. The same occurs with the controls without substrate (Figure 6), which do not show significant changes in SMA.

Finally, Table 2 summarizes the different percentages of SMA inhibition for each antibiotic used. In the case of CEF, the inhibitions were very low, and as shown previously, there were no statistically significant differences. Therefore, it can be concluded that this antibiotic does not affect the metabolism of anaerobic microorganisms in the doses tested. For OXY, significant inhibitions (over 20%) for higher antibiotic doses (5.0 mg/L and 10.0 mg/L) occurred. Since the other antibiotics do not present statistically significant differences, it can be assumed that their effect on microorganisms is minimal. For both FLO and PEN, all doses evaluated showed significant percentages of inhibition. However, PEN inhibited the microbes right from the beginning, with a percentage of 60.04%. This indicates that of the four antibiotics evaluated, PEN greatly affects the anaerobic processes, and in contrast, the CEF has no major impact.

Studies by Seija & Vignoli (2006) have shown that both CEF and PEN act by interrupting the synthesis of the pseudopeptidoglycan in the archaea. Pseudopeptidoglycan is a polymer that is responsible for giving rigidity to the cell walls of microbes and protecting the microbes from osmotic rupture in aquatic environments. Furthermore, PEN induces a self-destruct or autolytic effect on microorganisms. Although both antibiotics act in the same way, the above results demonstrate that a low concentration of PEN has a much greater inhibitory effect than a dose of CEF. For the latter antibiotic to achieve the same effect as the PEN, the applied dose should be much larger. From an environmental point of

Table 2 | Percentage of SMA inhibition

	Inhibition (%)			
Concentration (mg/L)	**CEF**	**OXY**	**FLO**	**PEN**
0.2	0.94	6.65	19.21	60.04
0.5	0.93	14.37	32.06	73.66
1.0	10.83	16.04	34.08	75.43
5.0	8.83	30.22	50.01	92.28
10.0	7.57	41.12	66.20	91.53

view, these results are important since both antibiotics have octanol-water partition coefficients (K_{ow}) that are much higher than those of OXY and FLO. The K_{ow} for PEN is 1.83 and for CEF it is 1.60, while for FLO it is −0.12 and for OXY it is −0.9. This indicates that both PEN and the CEF are hydrophobic antibiotics, and therefore, their distribution in living organisms occurs in the lipid layers. OXY and FLO are hydrophilic antibiotics, which means they enter and distribute themselves through the general diffusion porins.

Massé *et al.* (2000) reported that penicillin reduced CH_4 production in the anaerobic digestion of pig manure by 35% at mesophilic temperatures. Other studies have also shown that the production of CH_4 in anaerobic digesters was adversely affected by high concentrations of antibiotics (Li *et al.* 2008). Arikan *et al.* (2008) studied the fate of chlortetracycline (CTC), reporting that its concentration was reduced by ~75% during the period of 33 d digestion. More recently, Alvarez *et al.* (2010) reported inhibitory effects of OXY and CTC during the anaerobic digestion of pig manure.

According to the above results, and knowing that the behaviour of methane production over time corresponds to a first order kinetics reaction in which degradation depends on the substrate concentration, the kinetic constants (Table 3) were calculated.

$$\frac{dC}{dt} = -kC \text{ (First order kinetics)}$$

Table 3 shows that the highest constants are presented for CEF and OXY, while the lowest are for FLO and PEN, with PEN being the lowest. The low degradation velocity of PEN is associated with the aforementioned inhibitions.

Alvarez *et al.* (2010) calculated the first order constants for the anaerobic degradation of OXY and CTC, finding values of 0.052 d^{-1}, 0.045 d^{-1} and 0.058 d^{-1} for OXY concentrations of 10 mg/L, 50 mg/L and 100 mg/L, respectively. On the other hand, Arikan *et al.* (2006) reported a much lower value of 0.012 d^{-1} for OXY. In this study, constants with values of 0.0629 d^{-1}, 0.0646 d^{-1}, 0.0523 d^{-1}, 0.0523 d^{-1}, 0.0556 d^{-1} and 0.0540 d^{-1} were obtained for concentrations of 0.2 mg/L, 0.5 mg/L, 1.0 mg/L, 5.0 mg/L and 10.0 mg/L respectively, which are in agreement with values reported by Alvarez *et al.* (2010).

Figure 7 shows the variation in the percentage of COD removal for each antibiotic. According to the results analysed previously, CEF was the antibiotic that caused the lowest inhibition of microbes. This is reflected in the percentages of COD removal, which are all higher than 90%. The percentage of COD removal of the control (without antibiotics) was 96.72%. Almost the opposite occurred with penicillin, where removal percentages ranged from

Table 3 | First order kinetics constant for the antibiotics studied

Antibiotic	Concentration (mg/L)	K (d^{-1})	R^2
CEF	0.2	0.0935	0.8155
	0.5	0.0890	0.8194
	1.0	0.0884	0.8012
	5.0	0.0890	0.8193
	10.0	0.1129	0.9156
OXY	0.2	0.0629	0.7648
	0.5	0.0646	0.7675
	1.0	0.0523	0.8494
	5.0	0.0556	0.8606
	10.0	0.0540	0.9581
FLO	0.2	0.0553	0.8751
	0.5	0.0539	0.8652
	1.0	0.0496	0.9700
	5.0	0.0415	0.6778
	10.0	0.0350	0.8133
PEN	0.2	0.0308	0.8439
	0.5	0.0318	0.6706
	1.0	0.0265	0.7473
	5.0	0.0282	0.7193
	10.0	0.0209	0.6660

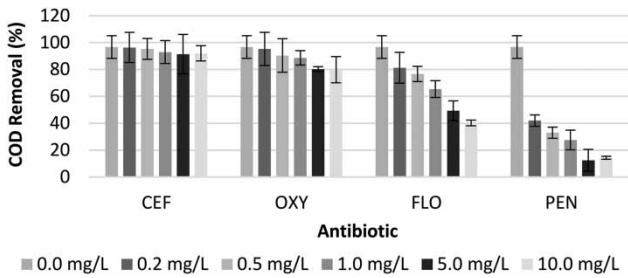

Figure 7 | COD removal percentages. Error bars indicate deviations of the data.

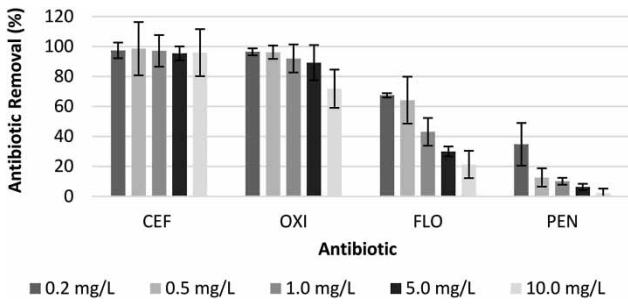

Figure 8 | Removal percentages of antibiotics.

41% for 0.2 mg/L to 14% for 10.0 mg/L. According to the statistical analysis, a statistically significant difference was found for four pairs, which included CEF with FLO and PEN, and OXY and FLO with PEN. The CEF, OXY and FLO presented differences compared with PEN, reinforcing the fact that PEN is the most inhibitory antibiotic in anaerobic processes.

Figure 8 shows removal percentages of each antibiotic. As with COD, the maximum removal occurred for CEF, with a percentage of up to 98%. In contrast, for PEN it was only possible to remove between 2.12% and 34.79%.

CONCLUSIONS

PEN demonstrated a high inhibition of anaerobic processes, affecting the production of methane even at low concentrations tested, opposite to CEF, which did not show an inhibitory effect on the methanogenic activity in any concentration case, showing that the mechanism inhibition of both antibiotics on the microorganisms is different even considering that both antibiotics belong to the same family of β-lactams. OXY only showed inhibition with higher concentrations and FLO showed inhibition at all the concentrations tested, but in a low proportion to the PEN. The inoculum and substrate validation showed that an inoculum concentration of 2.5 g VSS/L and a ratio of 1.5 g/L: 0.5 g/L: 0.5 g/L acetic acid: propionic: butyric, respectively, are adequate to achieve a good SMA of the order of 1.123 g COD-CH_4/g VSS d after acclimatization with acetic acid, which is essential to evaluate prior to the development of inhibition tests with antibiotics, to ensure adequate conditions for the anaerobic microorganisms present.

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