

IN VITRO ASSAY FOR ANTIMICROBIAL INTERACTION EVALUATION AND RISK ASSESSMENT OF ANTIMICROBIALS IN ANAEROBIC DIGESTION OF SWINE MANURE

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Introduction

The energy demand increase and recent new regulation for biomethane in Brazil have aroused new interests and perspectives for biogas from livestock wastes, especially swine manure. Brazil is the largest producer of animal protein and has perspective to keep growing in the next years, but to achieve this goal good practices of livestock waste management are required. The anaerobic digestion has become a common practice to treat the manure and also reduce production costs through energy recovery (Cherubini et al. 2015).

On the other hand, the use of veterinary drugs in livestock production and the occurrence in manure can lead to a number of concerns about interference in biogas production. The knowledge about inhibition effect and persistence of antimicrobials compounds in anaerobic digesters is important to take decision about the technology selection for manure treatment and to evaluate the environmental risk assessment in swine manure management.

Antibiotics are globally used in food-production animals as therapeutically (typically higher doses and administered individually or as group-treatment) to treat specific diseases and subtherapeutically (typically lower doses) for disease or infection prevention (Venglovsky, Sasakova, and Placha 2009). It is known that a significant fraction (30 to 90% - (Sarmah, Meyer, and Boxall 2006) of the antibiotics are excreted in the parent form or its metabolites (Schlüsener, Von Arb, and Bester 2006). In the excreted material, some metabolites are still biologically active and some inactive metabolites can potentially be transformed back to the bioactive parent compound under relatively mild conditions (Zhou et al. 2012).

Once tetracyclines have an antimicrobial wide spectrum, they are commonly used to treat infections in livestock. Unfortunately, the persistence of tetracyclines compounds in the manure can cause environment impact and influence the natural processes of biological treatment of waste (Prado, Ochoa, and Amrane 2009). Interference by veterinary drugs in the anaerobic digestion are diverse, such as excessive foaming in the reactor, decline in biogas productivity, accumulation of organic acids and imbalances in microbiology community (Shimada et al. 2011). For example, tetracycline in concentrations higher than 9 mg/L can reduce the methane production more than 50% in digestion of swine manure (Álvarez et al. 2010) and oxytetracycline reduce in 27% the biogas yield in manure from calves medicated for 5 days with 22 mg/kg/day (Arikan et al. 2006). Besides, the anaerobic digestion promotes the degradation of the antibiotic compounds and could mitigate the environmental

impact.

This work presents a proposal of laboratory procedure for anaerobic toxicity assay integrated with biodegradability assay, based on standard methods (ISO 13641, ISO 11734 and VDI 4630).

Material and methods

The antibiotics standards used in this study were purchased from Sigma-Aldrich (Germany) VETRANALTM quality, as follows: tetracycline hydrochloride, chlortetracycline hydrochloride, oxytetracycline hydrochloride and methacycline hydrochloride. As inhibitory control substance was used 3,5-dichlorophenol, also from Sigma-Aldrich (Germany).

The assays were performed according to VDI 4630 (2006). Batch experiments were performed in 250 mL glass reactors connected to 500 mL glass eudiometers. The reactors were prepared with 200 g (4.5 gVS) of the acclimated inoculum [mixture of anaerobic sludge from swine manure, UASB sludge from food industry and cow manure, prepared according Steinmetz et al. (2014)] and added 1 gVS of microcrystalline cellulose (20 µ, Sigma, Germany). To evaluate the antibiotic interference, 4 levels of concentration (x , $x \cdot 10^1$, $x \cdot 10^2$, $x \cdot 10^3$) of each tetracycline standard were spiked. The reactors were then sealed and stored at 37 °C until the establishment of stationary daily gas production rate (< 1% of the total amount produced). The production of gas was quantified on a daily basis by displacement of the sealant liquid level (DIN 38414-8, 1985) in the eudiometers. The dried biogas volume was determined by subtracting the water content based in the water vapour pressure, according to VDI recommendations. The dried biogas volume was then normalized to standard temperature and pressure i.e., 273 K and 1013 hPa, respectively. Specific biogas yield of each sample was estimated as the total biogas produced divided by the respective sample VS content. The results were normalized to the total biogas produced from negative controls prepared with inoculum only. All experiments were performed in triplicate.

The biogas inhibition was determined according ISO 13641-1 (2003) using the cumulative biogas after 72 h of incubation and using the maximal biogas production. The inhibition factor was estimated by application of the equation bellow:

$$I [\%] = \left(1 - \frac{V_t}{V_c}\right) \cdot 100$$

where I is the percentage inhibition, V_t is the biogas produced with the antibiotic in the selected time and V_c is the biogas produced in the control at the same time. The 10% and 50% inhibition concentration (IC₁₀ and IC₅₀) was estimated by regression analysis by plotting I against the logarithm of antibiotic concentrations.

The residual tetracycline content was evaluated by LC-MS/MS in a sample pool by mixture of 3 repetitions after de BMP assay (30 days). The sample preparation was done using SPE according Groth et al. (2015). The analysis was done by liquid chromatographic system Surveyor Plus coupled to mass spectrometer TSQ Quantum Access Max, both from Thermo Scientific (USA). The tetracycline separation was done using column Acclaim TM 120 C18 (150 mm x 4.6 mm, 5 µm - Thermo Scientific, EUA). The injection volume was 10 µL. The elution gradient had two phase: eluent A) formic acid 0.1% (v/v) in ultrapure water; eluent B) formic acid 0.1% (v/v) in acetonitrile; following the mixture system: 0-2 min isocratic 95% A and 5% B; 2-3 min

gradient to 25% of B; 3-7 min isocratic 25% of B; 7-12 min gradient to 50% of B; 12-15 min isocratic 50% of B; 15-16 min gradient to 100% of B; 16-18 min isocratic to 100% of B; 18-18,5 min gradient to 5% of B; 18,5-22 min isocratic to 5% of B, in 1.0 mL/min flow rate. Detection was preceded by electrospray ionization in positive mode. The quantification of tetracyclines was done by signal evaluation in “Selected Reaction Monitoring” mode. The precursor ions evaluated was (m/z) 461.1, 445.1, 479.1, 443.1 e 445.1 for oxytetracycline, tetracycline, chlortetracycline and doxycycline, respectively. The secondary ions evaluated were: m/z 426.3 (17 eV) and 443.3 (11 eV) for oxytetracycline; m/z 410.3 (18 eV) and 427.4 (11 eV) for tetracycline; m/z 462.3 (16 eV) and 444.3 (19 eV) for chlortetracycline and m/z 201.1 (33 eV) and 426.3 (13 eV) for methacycline.

Results and discussion

All treatments show low changes in pH (start test was 7.73 ± 0.04 ; after 30 days was 7.54 ± 0.07), confirming no limitations of alkalinity and the possible inhibitory effect are not linked to rapid changes in pH. The standard inhibiting substance 3,5-dichlorophenol of 150 mg/L presented $28 \pm 6\%$ inhibition after 72 h which is in accordance with ISO 13641-1(2003) for valid inhibition assays ($> 20\%$).

The Figure 1a shows the cumulative biogas production profile versus time, for the anaerobic digestion assays in the presence of tetracycline. There is a tendency of reduction in biogas production by increasing the concentration of antibiotics added. Tests with addition of tetracycline and chlortetracycline had similar profile, characterized by time increasing in the adaptation phase in the early days (from 1.2 days in the control to approximately 4 days in the highest concentration of antibiotic). Figure 1b shows the regression model obtained by the inhibition effects observed after 72 h and for the maximal biogas production (30 days). The same data evaluation was applied for the others antibiotics compounds. Table 1 show the estimative of IC₁₀ and IC₅₀ for biogas production at 72 hours of incubation and for the biogas yield. The IC₁₀ represent the minimum quantified level of inhibition.

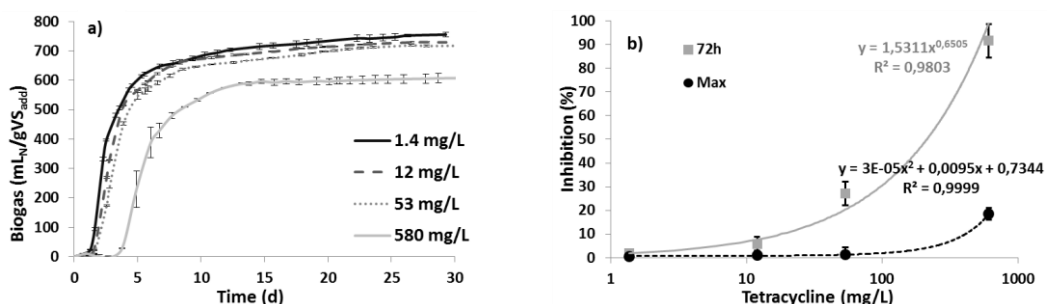


Figure 1. a) Cumulative biogas production profile from microcrystalline cellulose in the presence of variable concentrations of tetracycline; b) Regression equations for biogas inhibition.

Table 1. Estimation of IC₁₀ and IC₅₀ on biogas yield and tetracycline's compounds reduction after anaerobic assays.

Compound	IC ₁₀ mg/L	IC ₅₀ mg/L	Reduction* %
Tetracycline			
<i>I</i> _{72h}	19	219(207-231)	
<i>I</i> _{max}	414	1370(1339-1401)	46.0 - 96.5
Chlortetracycline			
<i>I</i> _{72h}	27	193(103-283)	
<i>I</i> _{max}	372	>> 2000	82.0 - 98.4
Oxytetracycline			
<i>I</i> _{72h}	68	495(361-629)	
<i>I</i> _{max}	287	1908(1354-2446)	76.6 - 98.7
Methacycline			
<i>I</i> _{72h}	18	142(75-208)	
<i>I</i> _{max}	127	>> 2000	57.1 - 97.6

Confidence interval in parenthesis is 95% based in standard error of regression.

*Reduction of initial concentration after 30 days of incubation.

In this case, by comparison of the IC₅₀ at 72 h, highest toxicity was observed for methacycline, in sequence chlortetracycline, tetracycline and finally oxytetracycline. But, according to the confidence interval is only possible to confirm that the toxicity of oxytetracycline was lower than the others. The IC₅₀ in biogas yield was significant in comparison between the initial gas productions in 72 h. In this case, tetracycline and oxytetracycline demonstrate similar level of inhibition. Similar profile has also identified in the methane production. The concentration of methane found in the tests were 55 ± 5% for tetracycline, 54 ± 5% chlortetracycline, 55 ± 4% oxytetracycline and 56 ± 4% methacycline. Therefore, based on the results from Table 1 it is possible to occur of acute inhibition in digester in full scale. For example, in pig manure occurrence of tetracycline 98,8 mg/L, oxytetracycline 354 mg/L, chlortetracycline 764.4 mg/L and methacycline 5.43 mg/L have been reported (Zhao, Dong, and Wang 2010; Pan et al. 2011; Chen et al. 2012).

On the other hand, the analysis of the residual concentration of veterinary drugs after the batch anaerobic assays indicates high degradation. In some experiments it was observed reduction of antibiotics over than 98%. These values agree with the data observed by Tong et al. (2012), that found reduction of 88.6-91.6% for tetracycline and 97.7-98.2% for chlortetracycline and by Turker et al. (2013) observed decrease of 55 to 70% for oxytetracycline.

Conclusions

The IC levels founded by the in vitro assay showed that is possible to occur of acute inhibition in anaerobic digestion of swine manure in full scale. The general inhibition effect in biogas production follows the order: methacycline > tetracycline ~ chlortetracycline > oxytetracycline. However, anaerobic digestion has been shown to be effective for the degradation of the compounds evaluated in the study and could help to reduce the environmental impact of veterinary drugs in the manure.

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