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Jonathan M. Withey[1,*], Samuel M. Mugo[1], Ting Zhou[1], Patrick M. Rosser[1], Tiejun Gao[2]

[1]*Department of Physical Sciences, MacEwan University, 10700 104 Avenue, Edmonton, Alberta, Canada, T5J 4S2*

[2]*Himark BioGas Inc., Agri-Food Discovery Place, F-83 6004 118 Street, Edmonton, Alberta, Canada, T6G 2E1*

**Correspondence to: 5-132B City Centre Campus, Department of Physical Sciences, MacEwan University, 10700 104 Avenue, Edmonton, Alberta, Canada, T5J 4S2*

E-mail: witheyj@macewan.ca.

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/jctb.4823

ABSTRACT

BACKGROUND: The fate and effect of hormone and antimicrobial residues in the manure of therapeutically treated cattle is of considerable concern because of the adverse effects of environmental loading of these chemicals. The objective of this study was to determine the depletion of tylosin (TYL), chlortetracycline (CTC), sulfamethazine (SMZ) and megestrol (MEG) using thermophilic anaerobic digestion (TAD).

RESULTS: Quantitative methods using HPLC-DAD-MS/MS analysis were developed to monitor the hormone and antimicrobials, spiked into manure obtained from unmedicated cattle. These manure samples were incubated for a 28-day period, during which hormone and antimicrobial depletion, and biogas production, was determined. Significant depletion of TYL, CTC, SMZ and MEG was demonstrated using TAD. Both TYL and CTC underwent complete depletion, while SMZ and MEG were each 80 % depleted by the 28th day of incubation. The presence of antimicrobials had no negative effects on process stability and caused no significant reduction in total methane production.

CONCLUSION: This process has demonstrated value in reducing the hormone and antimicrobial load to the environment, while not compromising economic value of the biogas production, and allowing use of post-digested biosolids as a regenerated fertilizer.

Keywords: anaerobic digestion, biogas, degradation, waste treatment and waste minimisation.

INTRODUCTION

There is growing public concern for the presence of hormones and antimicrobials in water and soil, and the possible pathways by which these substances can enter into human and animal food chains.[1,2] Of key concern is the routine administration of these substances in livestock farming and concentrated feedlot practices. Antimicrobials are used at both therapeutic and sub-therapeutic levels for disease prevention, control and treatment. They also serve to improve nutritional efficiency and promote growth rate. Hormones are administered as reproductive regulators and growth promoters. Recent estimates suggest that of the order of 16 million kilograms of hormones and antimicrobials are used annually in routine agricultural operations in North America (Animal Health Institute Website; http://www.ahi.org). It is estimated that up to 80 % of administered hormones and antimicrobials are excreted in unaltered form, or as partially metabolized derivatives that retain their pharmaceutical activity or are subject to retransformation to the parent compound.[3-6]

A 2011 survey conducted by the United States Department of Agriculture reported that there were 105 million head of cattle in North America, producing upwards of one billion kilograms of manure waste annually (United States Department of Agriculture: National Agricultural Statistics Service Website; http://www.nass.usda.gov). A significant portion of these cattle are raised in confined animal-feeding operations (CAFOs), where manure is land applied or composted.[1,7-10] A growing body of evidence suggests that hormones and antimicrobials can persist for prolonged periods in manure and, given the land application of cattle waste as a source of fertilizer, transfer to surface and groundwater is likely.[11,12] Antimicrobial residues in the environment are of concern due to the development of

resistance amongst native microbial communities in soil.[8,13] Hormones can act as endocrine disrupting chemicals (EDCs) which pose significant harmful impacts to wildlife, particularly birds and fish.[2,14-16]

Aerated lagoons, pit and pile storage, and composting are common manure-management strategies prior to disposal to the environment. Whilst the fate of antimicrobials under conditions of composting or in lagoons has been documented, there is limited information on the effect of anaerobic digestion (AD), an emerging manure management technology, on depletion of residual hormones and antimicrobials in manure and feedlot wastes.[1,2,8,17-19] AD is an environmentally friendly way to treat agricultural and livestock biowaste while extracting renewable energy in the form of methane-rich biogas, which can be used to generate heat, electricity, or compressed transportation fuel.[20] The AD technology reduces the bulk of biowaste by accelerating natural carbon/nitrogen cycles; the post-digested biomass can also be used as fertilizer. Recently, several studies have indicated that AD could inactivate pathogenic bacteria and viruses, depending on the digestion conditions and bacterial species.[21-23] It also has demonstrated efficacy in reducing greenhouse gas emissions, and odours, in addition to providing waste stabilization.[24] It is our hypothesis that inclusion of an AD step during manure treatment might, in addition to the aforementioned benefits, also degrade and inactivate the hormones and antimicrobials existing in cattle manure by exploiting the biological, chemical, and thermal components of the process. This would not only create additional value for post-digested manure as biofertilizer free of hormones, antimicrobials and pathogens, but also protect the environment from potential hormone and antimicrobial contamination.

The majority of studies of AD have used swine manure as the substrate, and focused exclusively on the inhibition of AD and, by extension, biogas production as a result of the higher concentrations of antimicrobials typically found in manure. Despite the existence of a considerable body of work, the results are inconsistent or even contradictory.[25] Few studies have been conducted on the fate and, specifically, the depletion of hormones and antimicrobials during AD. Of the limited information that does exist, dissipation half-lives under AD conditions have been shown to be less than 24 hours for tetracycline antimicrobials, while antimicrobials such as sulfamethazine show no detectable degradation.[26]

By determining the effect of three common cattle husbandry antimicrobials, and one hormone, this study was designed to expand the body of knowledge related to AD inhibition by antimicrobials and hormones in cattle manure, and to explore the depletion of such compounds during the AD process. Tylosin (TYL), chlortetracycline (CTC) and sulfamethazine (SMZ) represent the most widely used, broad spectrum antimicrobials administered at sub-therapeutic doses for cattle.[1] They also represent the structural diversity of different antimicrobial classes. Megestrol (MEG) is a synthetic gestagen.[27]

Figure 1. Structures of tylosin (TYL), chlortetracycline (CTC), sulfamethazine (SMZ) and megestrol (MEG).

The specific objectives were to determine whether thermophilic anaerobic digestion (TAD) can effect the depletion of these representative hormone and antimicrobial compounds, and whether these hormone and antimicrobial compounds have an inhibitory effect on biogas production.

EXPERIMENTAL

Chemicals and materials

TYL (CAS number 1401-69-0, $C_{46}H_{77}NO_{17}$, 916.10 g/mol, 8 mg/mL dissolved in aqueous 0.9% NaCl), CTC (CAS number 57-62-5, $C_{22}H_{23}ClN_2O_8$, 478.88 g/mol) and SMZ (CAS number 57-68-1, $C_{12}H_{14}N_4O_2S$, 278.33 g/mol), were purchased from Sigma-Aldrich (St. Louis, MO, USA). MEG (CAS number 3562-63-8, $C_{22}H_{30}O_3$, 342.47 g/mol) and formic acid were purchased from Fluka (St. Louis, MO, USA). Microcrystalline cellulose was purchased from Acros (CAS number 9004-34-6, NJ, USA). Acetonitrile, citric acid, sodium hydroxide, ethylenediaminetetraacetic acid (EDTA) and methanol were purchased from Fisher Chemical (Fair Lawn, NJ, USA). 13 mm syringe filters (0.2 µm PTFE membrane) were purchased from VWR (Radnor, PA, USA) and were used to filter samples prior to HPLC analysis. All water used in experiments was of Milli-Q quality.

Anaerobic digestate was freshly obtained from an active digester in an industrial biogas plant (Growing Power Hairy Hill, Vegreville, Alberta, Canada). The biogas plant operates three 10,400 m^3 thermophilic anaerobic digesters with 2.5 MW of electricity generation capacity. Each digester is configured with a continuous stirred-tank reactor (CSTR) and the operating temperature is 55 °C. Digesters receive up to 200 tonnes of cattle manure and other biowaste per day, derived from an adjacent feedlot that produces 400 tonnes of manure daily. The anaerobic digestate contains 8 – 10 % total solids (TS) and 78.32 ± 1.14 % volatile solids (VS) out of the total solid content, with a pH of 7.6 ± 0.13. Digestate was acclimatized in the lab digester for one week before it was used for experiments.

Control manure (free from the hormone and antimicrobials to be studied) was freshly collected from unmedicated dairy cattle at the Spring Creek Ranch (Vegreville, Alberta, Canada). Upon collection, the manure was stored at -20 °C. The control manure was tested using the developed SPE-LC-MS/MS method and confirmed to contain no detectable amounts of TYL, CTC, SMZ or MEG.

Extraction method for TYL, CTC, SMZ and MEG

2.0 g of control manure were spiked with TYL, CTC, SMZ and MEG (1000 mg/L stock solution, each prepared in 40:60 (v:v) acetonitrile/water) to prepare standards of 1.0, 10, 30, 50 and 100 mg/L of each analyte. Samples were thoroughly mixed before immediate extraction. The spiked manure was extracted twice with 10 mL of 8:2 (v/v) acetonitrile/0.25 M citric buffer (pH 4.0). After each extraction, the extracts were centrifuged (1400 RPM, 20 min); the supernatants were pooled and concentrated to remove the acetonitrile. 1 mL deionised water was then added to further dilute any remaining traces of acetonitrile. To prevent the chelating of antimicrobial standards with metals, 0.2 g EDTA was added to the extracts and vortexed for 30 seconds.

Solid phase extraction (SPE) was performed on a Grace Extract Clean^{TM} C18 SPE cartridge (500 mg/8 mL) (Grace Davison Discovery Science, Deerfield, IL, USA). The cartridge was pre-conditioned with 5 mL methanol followed by 5 mL deionised water. The supernatant of the extracts was then loaded into the cartridge, drained at a flow rate of 1 drop sec^{-1} and cleaned with 5 mL deionised water at 1 drop sec^{-1}. The samples were eluted using 10 mL of 9:1 (v/v) methanol/0.5 M citric buffer (pH 4.0). The eluates were then passed through a

syringe filter prior to HPLC-DAD-MS/MS analysis. The recovery of the compounds was calculated using the peak area of the individual compound after extraction and the peak area of the compound with the same concentration that was dissolved in eluent.

HPLC-DAD-MS/MS analysis

Chromatographic separations were performed on a Thermo LCQ fleet ion trap HPLC-DAD-ESI-MS system (Thermo Scientific, San Jose, California, USA) consisting of an LC pump, an autosampler, a 6-port injection valve with a 20 µL injection loop, a DAD detector (diode array detector), an ESI (electrospray ionization) source and an ion trap mass spectrometry (MS) detector. The temperature of the column oven was set at 30 °C. The temperature of the autosampler tray was set at 6 °C to prevent depletion of the hormone and antimicrobials. An Agilent Zorbox Eclipse PAH RPLC column (250 × 3.0 mm I.D., 5 µm) (Agilent, Santa Clara, CA, USA) was used for the separation.

The separation of the one hormone (MEG) and three antimicrobials (TYL, CTC, SMZ) was achieved by an HPLC gradient method. Solvent A was acetonitrile and solvent B was 0.1 % formic acid in H_2O. The gradient started with 20 % A, increased to 55 % within 5 min, to 95 % within 1 min, was kept at 95 % for 5 min, returned to initial composition within 2 min, and equilibrated within 3 min for a total run time of 15 min. The mobile phase flow rate was 500 µL/min. The analytes were detected in tandem by DAD and mass spectrometry. The DAD detection range was set from 250 to 400 nm. Individual MS tuning for each hormone and antimicrobial was done by infusing a 10 mg/L standard with a flow rate of 10 µL/min into an individual mobile phase at 500 µL/min. Each mobile phase was chosen based on the composition when the individual standard was eluted. All the analytes were detected in

positive ESI mode. The Thermo LCQ fleet ion trap system was run in collision induced mode (MS^2) for confirmative identification and increased sensitivity. The precursor and product ions used for TYL, CTC, SMZ and MEG identification and quantitation are listed in table 1, along with associated collision energies.

Table 1. Tandem MS settings for each hormone and antibiotic.

Anaerobic digestion

The experiment was set up in a lab-scale digester in which four replicates of the assay were conducted using 250 mL glass digester bottles. The bottle contents were designed to mimic common AD cattle effluent and contained a mixture of control manure, sterile water, acclimatized thermophilic anaerobic digestate (as inoculum), and hormone and antimicrobial standards (concentration of 20 mg/l for each standard). Bottles were prepared by first mixing 150 g of cattle manure with sterile water at 1:1 (w/v) to generate a slurry, and then spiking the slurry with TYL, CTC, SMZ and MEG standards to give 100 mg/L of each compound in the slurry. 10 mL of this spiked manure slurry and 40 mL of digestate inoculum were added to each digester bottle (group 1) for a final volume of 50 mL, leaving 200 mL for headspace gas collection. Digester bottles were sealed with butyl rubber septa and purged with pure nitrogen gas to resume anaerobic conditions. Manure + water (group 2), and water-only controls (at both 55 °C and 22 °C; group 3 and group 4, respectively) containing 20 mg/l of each standard were also prepared (four replicates each). Additional biogas production controls were also set up, including TAD inoculum plus 10 mL of water; and TAD inoculum plus 10 mL water and 0.6 g crystalline cellulose (as a nutrient source for the microbes). The complete, detailed experimental design is shown in table 2. All digester bottles were placed in

an incubator with a temperature setting of 55 °C and gently mixed by hand daily. Sampling was carried out at day 0, 2, 7, 14, 21 and 28.

Table 2. Experimental design for depletion of hormones and antimicrobials using TAD process.

During the TAD process, biogas samples were taken from the headspace of the bottle at day 2, 7, 14, 21 and 28 from the TAD plus hormone/antimicrobials, TAD control and TAD plus cellulose control, respectively. After depressurization of the digester bottles, 20 mL of biogas was drawn using a syringe, injected into pre-vacuumed exetainers (Labco, Houston, TX) and analyzed by gas chromatography (Varian 450-GC) for methane and carbon dioxide composition. Biogas production was calculated after normalization and expressed as accumulated biogas production during the period of study.

For extraction of the samples containing TAD and spiked manure slurry (group 1 and group 2), 5 mL aliquots from each digester bottle at different sampling time intervals were transferred to a 50 mL centrifuge vial. Each aliquot was extracted twice using the developed method outlined previously. For the samples containing no TAD or manure slurry, 5 mL aliquots from each control bottle at different sampling time intervals were drawn and directly loaded into a C18 SPE cartridge at a flow rate of 1 drop sec^{-1}. The samples were eluted with 10 mL of 9:1 (v/v) methanol/0.5 M citric buffer (pH 4.0). All eluates were then passed through a syringe filter prior to HPLC-DAD-MS/MS analysis.

The depletion efficiency, as a function of time, of the TYL, CTC, SMZ and MEG standards was determined using the peak area of the day n standard and the peak area of the day 0

standard. Statistical analysis was performed on the effect of antimicrobials on methane production between TAD spiked with selected hormone and antimicrobial standards, and the TAD control. The Student's t-test was used at different sampling time intervals, and for comparison of cumulative methane production during the whole period of the experiment using the Chi-square test. The significance level was set at 95% ($p < 0.05$).

RESULTS AND DISCUSSION

Extraction efficiency and recovery of the compounds from manure

Extraction efficiencies and recovery of the selected hormone and antimicrobials from manure slurry using our extraction method are shown in table 3. Well-resolved chromatograms and acceptable recoveries of TYL, CTC, SMZ and MEG were achieved, which illustrated the applicability of the developed extraction method.

Table 3. Recovery of 1.0 mg/L hormone and antimicrobial standards spiked in control manure slurry.

Calibration curves of selected antimicrobial and hormone standards were generated by subjecting the analytes to the validated extraction process. Five concentrations of the hormone and antimicrobial standards at 1.0, 10, 30, 50 and 100 mg/L respectively were spiked into 2.0 g of control manure slurry, extracted, and subsequently analyzed by HPLC-DAD-MS/MS. Linear calibration curves were obtained for all standards up to the 100 mg/L range ($R^2 = 0.991$, 0.991 and 0.993 for TYL, SMZ and MEG, respectively), with the exception of CTC, which performed a linear response up to 50 mg/L ($R^2 = 0.996$).

Fate of TYL, CTC, SMZ and MEG in thermophilic anaerobic digestion and different controls

Different levels of depletion of the spiked hormone and antimicrobials were observed in this study (Figure 2). To calculate the percentage of depletion in different experimental settings at day 0, the MS peak area of the hormone and antimicrobial standards spiked in the water control at 22 °C was used as the baseline. In the case of groups 1 and 2, significant depletion of the parent antimicrobials (TYL, CTC and, for group 1 only, SMZ) appeared to occur during day zero. Such an occurrence has been reported previously for CTC and oxytetracycline (OTC).[11]

Figure 2. Depletion profiles of 20 mg/L hormone/antimicrobials under various conditions (see table 2): (a) tylosin; (b) chlorotetracycline; (c) sulfamethazine; (d) megestrol.

The depletion profiles of the selected hormone and antimicrobials at different experimental settings demonstrated clearly that both TYL and CTC have significant thermal lability. During the 28-day study period, TYL and CTC underwent less than 40 % depletion at ambient temperature (22 °C) while at least 80 % depletion was observed at 55 °C with or without the TAD/manure component within the first few days of the study. Complete CTC depletion was observed by day 14 and 90 % TYL depletion by day 28 in the water control group at 55 °C. By contrast, SMZ and MEG showed much greater thermal stability. There was no significant depletion (< 20 %) observed, nor variation in depletion between ambient and elevated temperatures in the control groups. During the 28-day study period, SMZ was depleted less than 20 % in the water control at both 55 and 22 °C. No observed depletion of

MEG occurred in the water control at either 55 or 22 °C, or in the manure plus water control at 55 °C.

Interestingly, the depletion profile of SMZ in the water plus manure control demonstrates the microbial effect on selected antimicrobials in comparison with the water control at 55 °C. SMZ underwent little appreciable depletion in the absence of manure, but reached almost 80 % depletion in the presence of manure by the end of the study. The manure contains a consortium of microbes similar to the well-established ones found in active anaerobic digestion. This was evident in the biogas production associated with that group. Methane production commenced after incubation in anaerobic conditions at 55 °C for 21 days (1.028 Nml/g VS, average) and increased to 6.872 Nml/g VS at day 28 because the initial mass of manure (~ 1.3 g) with microbes was small in each digester bottle and it took time to acclimatize and propagate for methane production.

Several studies have reported the importance of microbial depletion in the presence of manure, a factor involved in composting effects on antimicrobials in feedlot manure.[1,8] Such microbial activity appears not only to affect both the rate and extent of depletion of SMZ in particular, but also to accelerate the extent of TYL depletion as demonstrated in the current study. Over 80 % depletion of TYL was achieved almost immediately in the presence of manure and elevated temperatures while the same level of depletion was seen in water without manure only on day 21. The depletion profile for CTC in the manure plus water control group at 55 °C appeared anomalous in comparison with the active TAD group, and the water without manure at elevated temperature. Some substances in manure may inhibit depletion of CTC or interfere with analysis under the suboptimal anaerobic digestion process. The reason is still unclear.

Of note, the best depletion profiles for all four selected hormone and antimicrobials were observed in the well-established and active thermophilic anaerobic digestion group. TYL and CTC underwent essentially complete depletion by day 7 of the TAD process. This represents a moderate increase in both rate and extent of depletion relative to the manure plus water and water only groups at the same temperature. Additionally, there was a small increase in the extent of depletion of SMZ relative to the manure plus water (group 2). However, depletion of SMZ in both groups showed a similar pattern and reached only 80 % of depletion during this 28-day study. This indicated that TAD could destroy up to 80 % of the parent form of SMZ a day or two following establishment of a healthy digestion process, with the remaining 20 % likely remaining resistant to the TAD process. These observations compare favourably with the limited information that exists in the literature, where complete degradation of TYL has been observed in as little as 4 days, and with no reported degradation of SMZ under AD conditions.[26] Also noteworthy was the behaviour of MEG and its dynamic depletion under TAD. This synthetic hormone was completely stable under all other conditions in this study, exhibiting no depletion at elevated temperatures or in the presence of limited amounts of microbes. However, under active and well-established TAD, almost 80 % of the original dose of MEG was depleted during a 28-day digestion process, with a trend towards a complete depletion if the batch TAD digestion had been extended. This is particularly significant given the environmental persistence of MEG and its structural analogues,[28,29] and clearly illustrates the capability of TAD for the depletion of MEG, with the broader applicability to other hormones (both exogenous and endogenous) currently under study.

Effect of TYL, CTC, SMZ and MEG on biogas production

Batch assays were incubated at 55 °C for 28 days. Cumulative methane production, and carbon dioxide production in each assay is shown in Figure 3.

Figure 3. Biogas profiles in the executed assays: cumulative methane production, and cumulative carbon dioxide production.

Biogas production was measured during the study period for the following: a TAD control; a TAD plus cellulose control, and; TAD plus the hormone and antimicrobials. There was no obvious lag phase in biogas production in TAD containing the hormone/antimicrobials. However, methane production was lower in the TAD plus hormone/antimicrobials group than that in the TAD and TAD plus cellulose controls between day 3 and 7 (50.139 ± 3.589 versus 77.916 ± 5.278 and 75.023 ± 31.991 normalized ml (Nml) / g volatile solid (g VS), $p <$ 0.0001, t-test), and between day 8 and 14 (23.893 ± 3.968 versus 28.594 ± 3.589 2.007 and 31.904 ± 1.233 Nml/g VS, $p < 0.004$, t-test). Inhibition of methane production of TAD at the current doses of 20 mg/L each of CTC, TYL and SMZ in the mixture was observed between day 3 and 7 (35.6 %) and day 8 and 14 (16.4 %) in this study. There was no significant reduction of methane production in TAD containing CTC, TYL and SMZ observed from day 15 to 21 and day 22 to 28 ($p = 0.82$ and 0.31). For the entire period of the study, total cumulative methane production was 156.61 and 134.18 NmL/g VS for the TAD control and TAD containing the hormone and antimicrobials. There was no statistically significant difference between the two groups in terms of total methane production ($p = 0.06$, chi-square test). In fact, increased cumulative methane production was observed during day 15 to 28 for TAD with the hormone and antimicrobials (21.77 Nml /gVS) versus the TAD control (13.34 Nml/g VS). This could explain why there is no significant impact on total methane production.

Meanwhile, the carbon dioxide productivity was also inhibited in TAD with the hormone and antimicrobials (cumulative CO_2 116.2 NmL/g VS), compared to the TAD control (164.1 NmL/g VS). These results demonstrated that the three antimicrobials under study did not only inhibit methanogens, but also slowed down catalytic and metabolic activities of the entire consortium of microorganisms that exist in an active TAD process. The combined effects of TYL, CTC, and SMZ at 20 mg/L level caused only a moderate reduction of total biogas production during the 28-day period.

Previous studies have reported varied level of inhibition of antimicrobials on biogas production during anaerobic digestion of pig and cow manure, albeit in the presence of CTC and oxytetracycline (OTC) only (table 4). However, a broad disagreement is observed in the literature regarding the inhibitory effect of CTC and OTC. At an extreme, Lallai et al. observed no inhibitory effect with OTC concentrations as high as 250 mg/L,[30] whilst Arikan et al. observed a 27 % reduction in methane production with the lowest dose of 3.1 mg/L OTC.[7] The majority of data suggests inhibition of methane production in the presence of CTC and OTC, with the effect of CTC tending to be more pronounced.

Table 4. Effect of CTC and OTC on methane production during anaerobic digestion.

With the current study demonstrating only a modest inhibition of biogas production, the energy and economic value of the biogas is not significantly compromised.

CONCLUSIONS

TAD has been shown to be effective at depleting the selected hormone and antimicrobials. Elevated temperature and anaerobic microorganisms acted synergistically to achieve the best depletion, particularly for the hormone MEG. Though the mode of depletion of hormones and antimicrobials is largely unknown, it is anticipated that the synergistic effect of microbial activities, high pH, elevated temperature and presence of other small molecules will destabilize the selected hormone and antimicrobials leading to degradation. However, these experiments do not resolve whether hormone and antimicrobial depletion is effected by degradation, mineralization, or binding to the manure matrix. Moreover, the relative contributions of individual factors that occur during thermophilic anaerobic digestion, and the interactions between these factors, are not distinguished. These factors (elevated temperature, biologically transformed organic material, high biological activity) may impact the levels of extractable hormones and antimicrobials.

Under the concept of a fully integrated utilization of biowaste to energy, with hormones and antimicrobials being readily depleted in a biological and environmentally friendly way, additional value of post-digested biosolids as a regenerated organic fertilizer could be realized. It is important to mention, in concluding, that much research has been done on dissipation of veterinary antimicrobials (VA) using spiked manure samples. There is some evidence from the literature that dissipation in manure from animals administered VA is different than that from spiked samples. The fact that the VA are fed to the animals in their diets and pass through the microbial conditions associated with the rumen somehow alters dissipation rates (faster or slower, is open to question) compared to when VA that have been added to manure post-excretion. These observations should be considered something of a caveat to the current findings, with a full investigation into dissipation in manure from animals administered VA currently underway in our laboratory.

ACKNOWLEDGEMENTS

This work was supported by generous funding from Grant MacEwan University, the Alberta Association of Colleges and Technical Institutes (AACTI), the Natural Sciences Engineering Research Council (NSERC) of Canada, and Biowaste-to-energy for Canada: an integration initiative (BECii). Funding from AVAC Ltd. provided specific support for depletion experiments conducted in Himark's R&D laboratory. The authors thank Himark BioGas Inc. for providing technical support in the depletion experiments and manure/TAD inoculums. Support from the facilities and staff of Agri-Food Discovery Place and Dairy Research and Technology Centre (University of Alberta, Edmonton, Alberta, Canada) is also acknowledged.

REFERENCES

1 Sarmah AK, Meyer MT and Boxall A, A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antimicrobials (VAs) in the environment. *Chemosphere* **65**:725-759 (2006).

2 Zheng W, Yates SR and Bradford SA, Analysis of steroid hormones in a typical dairy waste disposal system. *Environ Sci Technol* **42**:530-535 (2008).

3 Boxall ABA, Kolpin DW, Halling-Sorensen B and Tolls J, Are veterinary medicines causing environmental risk? *Environ Sci Technol* **37**:286A-294A (2003).

4 Hanselman TA, Graetz, DA and Wilkie AC, Manure-borne estrogens as potential environmental contaminants: a review. *Environ Sci Technol* **37**:5471-5478 (2003).

5 Jjemba PK, The potential impact of veterinary and human therapeutic agents in manure and biosolids on plants grown on arable land: a review. *Agric Ecosyst Environ* **93**:267-278 (2002).

6 Kumar K, Gupta SC, Chander Y and Singh AK, Antimicrobial use in agriculture and its impact on the terrestrial environment. *Adv Agron* **87**:1-54 (2005).

7 Arikan OA, Sikora LJ, Mulbry W, Khan SU, Rice C and Foster GD, The fate and effect of oxytetracycline during the anaerobic digestion of manure from therapeutically treated calves. *Process Biochem* **41**:1637-1643 (2006).

8 Arikan OA, Mulbry W, Ingram D and Millner P, Minimally managed composting of beef manure at the pilot scale: Effect of manure pile construction on pile temperature profiles and on the fate of oxytetracycline and clorotetracycline. *Bioresour Technol* **100**:4447-4453 (2009).

9 Bao Y, Zhou Q, Guan L and Wang Y, Depletion of chlorotetracycline during composting of aged and spiked manures. *Waste Manage* **29**:1416-1423 (2009).

10 Dolliver H, Gupta S and Noll S, Antimicrobial depletion during manure composting. *J Environ Qual* **37**:1245-1253 (2008).

11 Álvarez JA, Otero L, Lema JM and Omil F, The effect and fate of antimicrobials during the anaerobic digestion of pig manure. *Bioresour Technol* **101**:8581-8586 (2010).

12 Lindsey ME, Meyer M and Thurman EM, Analysis of trace levels of sulfonamine and tetracycline antimicrobials in groundwater and surface water using solid-phase extraction and liquid chromatography-mass spectrometry. *Anal Chem* **73**:4640-4646 (2001).

13 Chee-Sanford JC, Aminiov RI, Krapac IJ, Garrigues-Jean NG and Mackie RI, Occurrence diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. *Appl Environ Microbiol* **67**:1494-1502 (2001).

14 Khanal SK, Xie B, Thompson ML, Sung S, Ong S-K and van Leeuwen J, Fate, transport, and biodegradation of natural estrogens in the environment and engineered systems. *Environ Sci Technol* **40**:6537-6546 (2006).

15 Kolodziej EP, Harter T and Sedlak DL, Dairy wastewater, aquaculture, and spawning fish as sources of steroid hormones in the aquatic environment. *Environ Sci Technol* **38**:6377-6384 (2004).

16 Yamamoto A, Kakutani N, Yamamoto K, Kamiura T and Miyakoda J, Steroid hormone profiles of urban and tidal rivers using LC/MS/MS equipped with electrospray ionization and atmospheric pressure photoionization sources. *Environ Sci Technol* **40**:4132-4137 (2006).

17 Arikan OA, Rice C and Codling E, Occurrence of antimicrobials and hormones in a major agricultural watershed. *Desalination* **226**:121-133 (2008).

18 Ermawati R, Morimura S, Tang Y, Liu K and Kida K, Depletion and behavior of natural steroid hormones in cow manure waste during biological treatments and ozone oxidation. *J Biosci Bioeng* **103**:27-31 (2007).

19 Frear C, Mitchell SM, Ullman, JL, Teel, AL and Watts RJ, The effects of the antibiotics ampicillin, florenicol, Sulfamethazine, and tylosin on biogas production and their degradation efficiency during anaerobic digestion. *Bioresour Technol* **149**:244-252 (2013).

20 US EPA, 2006. Market opportunities for biogas recovery systems – A guide to identifying candidates for on-farm and centralized systems. EPA-430-8-06-004. US Environmental Protection Agency, Washington, DC.

21 Gao TJ, Bowlby E, Tong YP, Wu JTY, Wong L, Pang XL and Li X, Evaluation of the matrix effect of thermophilic anaerobic digestion on inactivation of infectious laryngotracheitis virus using real-time PCR and viral cell culture. *Bioresour Technol* **110**:692-696 (2012).

22 Smith SR, Lang NL, Cheung KHM and Spanoudaki K, Factors controlling pathogen destruction during anaerobic digestion of biowastes. *Waste Manage* **25**:417-425 (2005).

23 Wagner AO, Gstraunthaler G and Illmer P, Survival of bacteria pathogens during the thermophilic anaerobic digestion of biowaste: laboratory experiments and in situ validation. *Anaerobe* **14**:181-183 (2008).

24 Frear C, Liao W, Ewing T and Chen S, Evaluation of co-digestion at a commercial dairy anaerobic digestor. *Clean: Water, Air, Soil* **39**:97-704 (2011).

25 Lu X, Zhen G, Liu Y, Hojo T, Estrada AL and Li Y-Y, Long-term effect of the antibiotic cephalexin on methane production during waste activated sludge anaerobic digestion. *Bioresour Technol* **169**:644-651 (2014).

26 Mitchell SM, Ullman JL, Teel AL, Watts RJ and Frear C, The effects of the antibiotics ampicillin, florfenicol, sulfamethanzine, and tylosin on biogas production and their degradation efficiency during anaerobic digestion. *Bioresour Technol* **149**:244-252 (2013).

27 Scanlon PF and Burgess TD, Subcutaneous and oral applications of progestogens for control of estrus in heifers. *Can J Anim Sci* **52**:767-769 (1972).

28 Liu ZH, Ogejo JA, Pruden A and Knowlton KF, Occurrence, fate and removal of synthetic oral contraceptives (SOCs) in the natural environment: a review. *Sci Total Environ* **409**: 5149-61 (2011).

29 Schiffer B, Totsche KU, Jann S, Kögel-Knabner I, Meyer K and Meyer HH, Mobility of the growth promoters trenbolone and melengestrol acetate in agricultural soil: column studies. *Sci Total Environ* **326**:225-37 (2004).

30 Lallai A, Mura G and Onnis N, The effects of certain antimicrobials on biogas production in the anaerobic digestion of pig waste slurry. *Bioresour Technol* **82**:205-208 (2002).

31 Sanz JL, Rodríguez N and Amils R, The action of antimicrobials on the anaerobic digestion process. *Appl Microbiol Biotechnol* **46**:587-592 (1996).

32 Loftin KA, Adams CD, Surampalli R and Mormile MR, Inhibition of microbial metabolism in anaerobic lagoons by selected sulfomamides, tetracyclines, lincomycin, and tylosin tartrate. *Environ Toxicol Chem* **24**:782-788 (2005).

Table 1. Tandem MS settings for each hormone and antibiotic.

standard	precursor ion m/z	product ion m/z	collision energy (%)
SUL	279.1	124, 186	30
CTC	479.1	462, 444	35
TYL	916.3	772.2	23
MEG	385.1	267.2, 325.1	30

Table 2. Experimental design for depletion of hormones and antimicrobials using TAD process.

content of digester bottle (n=4)	temp. (°C)	group #	TAD (mL)	manure (mL)	water (mL)
antimicrobials/hormone + manure + TAD	55	1	40	10	0
antimicrobials/hormone + manure + water	55	2	0	10	40
antimicrobials/hormone + water	55	3	0	0	50
antimicrobials/hormone + water	22	4	0	0	50

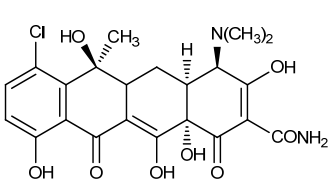
Table 3. Recovery of 1.0 mg/L hormone and antimicrobial standards spiked in control manure slurry.

standard	recovery (%) (n = 3)
TYL	96.7 ± 2.5
CTC	90.0 ± 11.3
SMZ	83.7 ± 8.4
MEG	81.4 ± 2.4

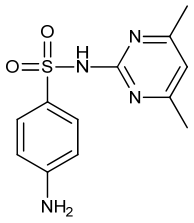
Table 4. Effect of CTC and OTC on methane production during anaerobic digestion.

compound	concentration (mg/L)	CH_4 production decrease (%)	reference
OTC	125	No inhibition	Lallai et al.[30]
	250	No inhibition	
OTC	3.1	27	Arikan et al.[7]
CTC	5	20	Sanz et al.[31]
	40	50	
	152	80	
OTC	1	2	Loftin et al.[32]
	5	5	
	25	7	
CTC	1	32	Loftin et al.[32]
	5	33	
	25	44	
OTC and CTC	10	45	Álvarez et al.[11]
	50	57	
	100	64	
TYL, CTC, SMZ and MEG	20	35 (day 3 to 7), 16 (day 8 to14)	This work

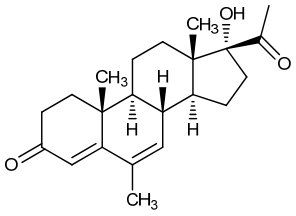
Figure 1. Structures of tylosin (TYL), chlortetracycline (CTC), sulfamethazine (SMZ) and megestrol (MEG).



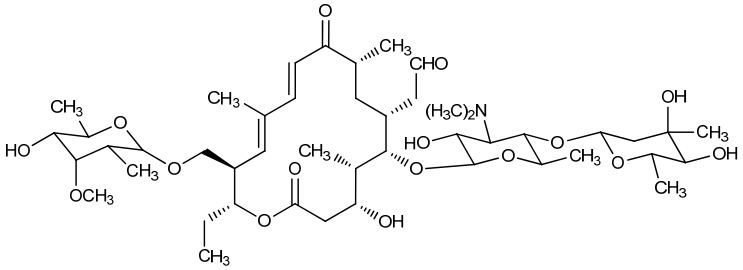
chlorotetracycline



sulfamethazine



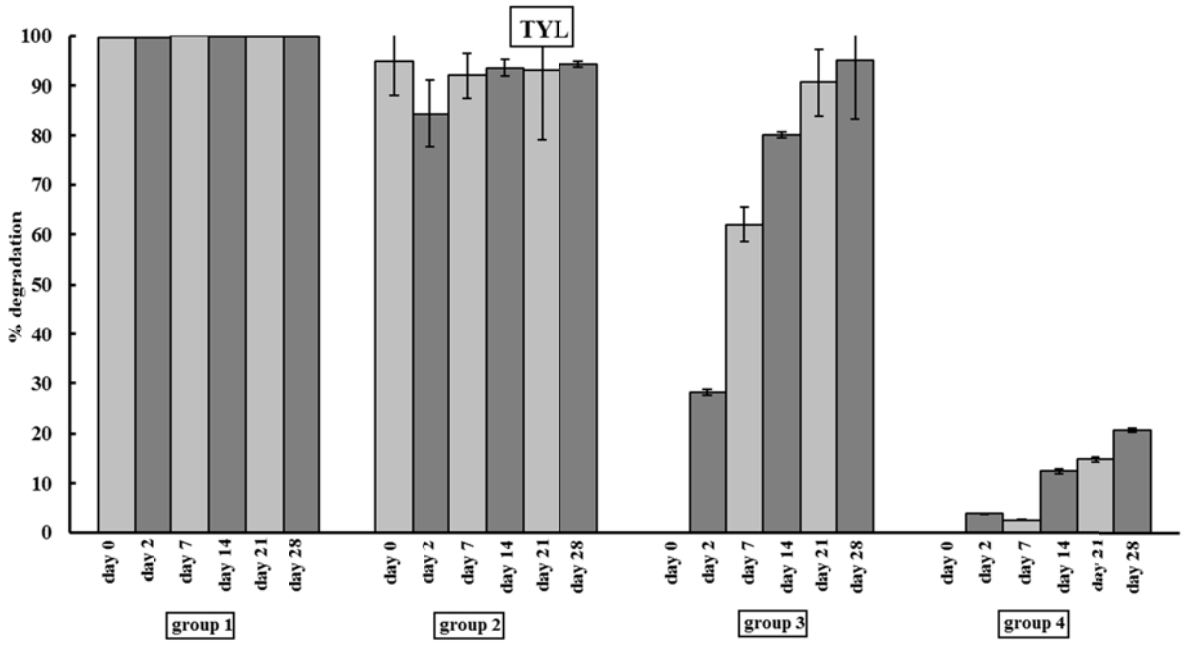
megestrol



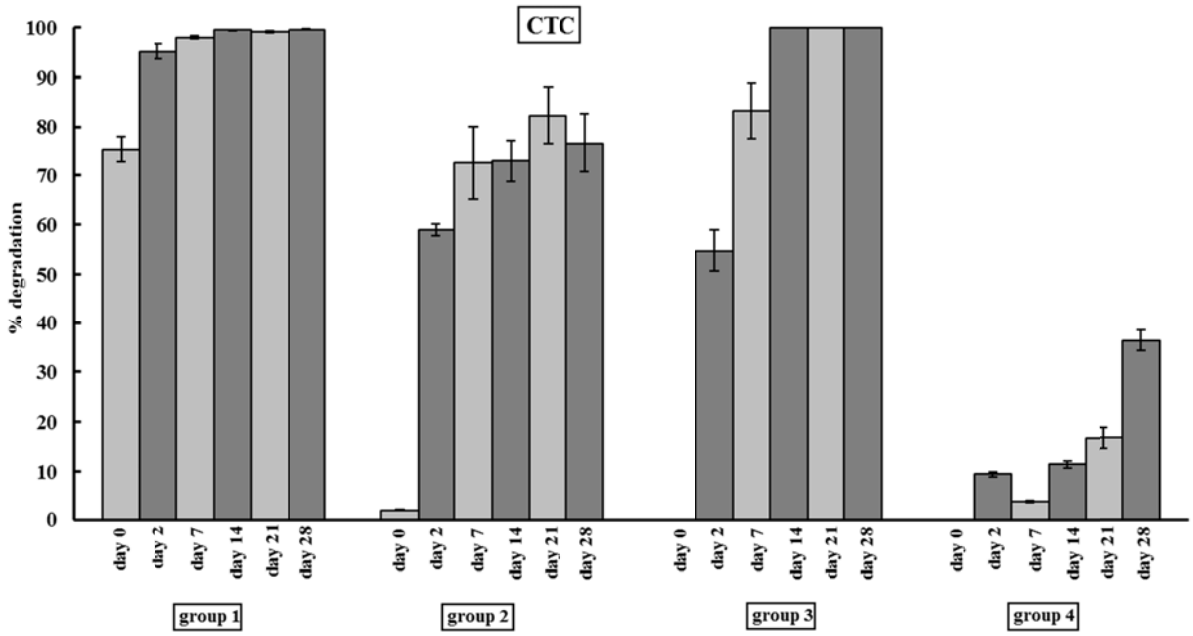
tylosin

Figure 2. Depletion profiles of 20 mg/L hormone/antimicrobials under various conditions (see table 2): (a) tylosin; (b) chlorotetracycline; (c) sulfamethazine; (d) megestrol.

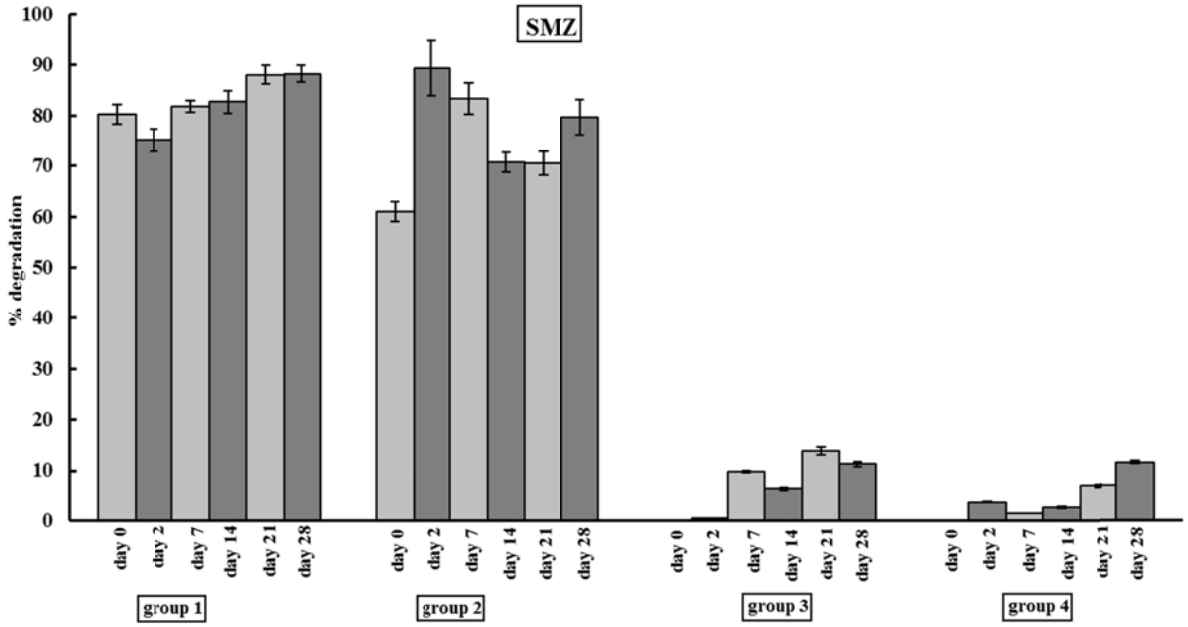
(a)



(b)



(c)



(d)

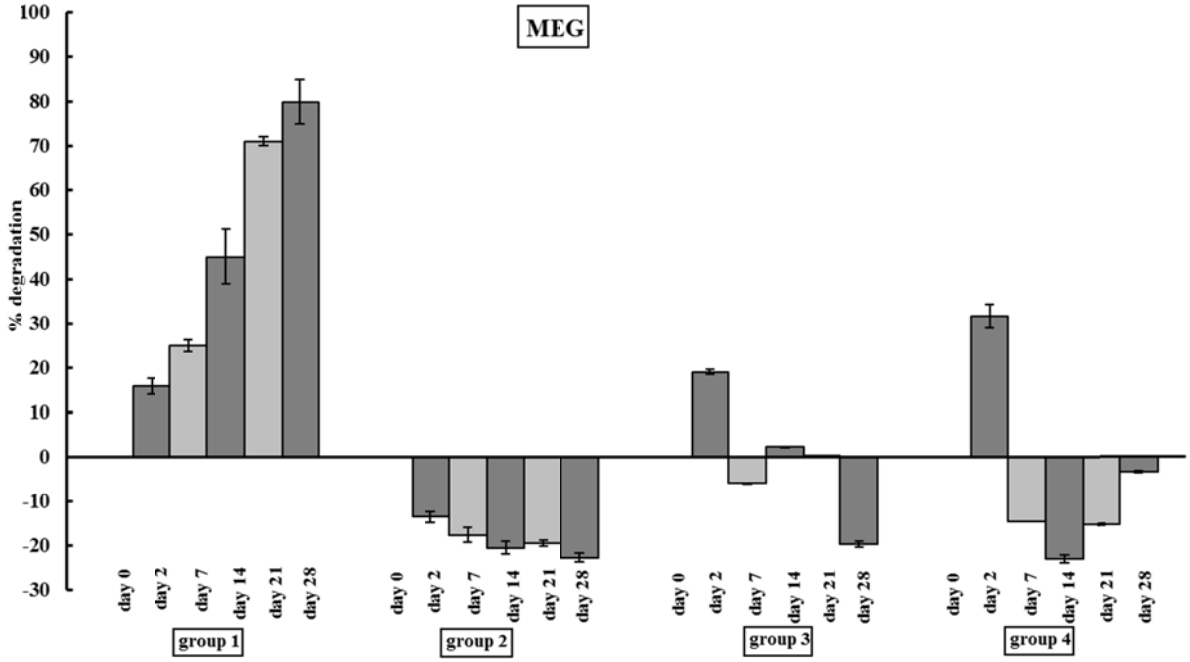


Figure 3. Biogas profiles in the executed assays: cumulative methane production, and cumulative carbon dioxide production.