The fate and effect of oxytetracycline during the anaerobic digestion of manure from therapeutically treated calves

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Received 27 October 2005; received in revised form 10 March 2006; accepted 14 March 2006

Abstract

The fate of antibiotic residues in the manure of treated animals is of considerable concern because of the potential development of antibiotic-resistant bacteria in the environment and because of the effect of these residues on manure treatment systems. The objective of this study was to determine the fate and effect of oxytetracycline (OTC) during the anaerobic digestion of manure from medicated calves. Five beef calves were medicated for 5 days with 22 mg/kg/day of OTC. Manure samples collected from calves prior to and after medication were diluted five-fold with water, loaded into replicate 1.2 l anaerobic digesters and incubated at 35 \degree C. OTC levels in the manure slurry decreased from 9.8 \pm 0.1 to 4.1 \pm 0.1 mg/l in 64 days (59% removal) yielding a calculated value half-life for OTC of 56 days. Levels of the OTC epimer 4-epi-oxytetracycline increased gradually from 0.55 \pm 0.03 mg/l at the start of experiment to 1.3 \pm 0.1 mg/l on day 27 and then decreased to 0.84 \pm 0.04 mg/l on day 64. Levels of two other OTC metabolites (\alpha-apo-oxytetracycline and \beta-apo-oxytetracycline) decreased or remained unchanged during the anaerobic digestion process. Cumulative biogas production was 27% lower from digesters containing manure from medicated calves relative to that from digesters containing unmedicated manure. However, the presence of OTC did not show other negative effects on process stability as there were no significant differences in biogas methane content or in reductions of volatile solids and soluble organic carbon.

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Keywords: Oxytetracycline; Anaerobic Digestion; Manure; Antibiotic; Fate; Inhibition

1. Introduction

Oxytetracycline (OTC) is a common antibiotic used in livestock animals (including cattle, swine, poultry and fish) for prophylactic and therapeutic treatment, and as a growth promoter due to its broad spectrum of activity and low cost. 4-epi-Oxytetracycline (EOTC), \alpha-apo-oxytetracycline (\alpha-Apo-OTC) and \beta-apo-oxytetracycline (\beta-Apo-OTC) are degradation products of OTC (Fig. 1). In 1998, 500,000 kg of OTC (65,000 kg for cattle) were used in livestock for non-therapeutic purposes in the United States [2]. Recent results in our laboratory have shown that approximately 23% of the OTC fed to calves is excreted in manure [3]. The widespread use and relative persistence of OTC have lead to its detection in soil [4] and surface waters [5].

The release of antibiotics into the environment is of considerable concern because persistent antibiotic residues may lead to development of antibiotic-resistant bacteria [6]. In addition, residues of antibiotics or metabolites in manure can have negative effects on treatment systems such as anaerobic digesters [7] and nitrifying systems [8].

Anaerobic digestion is an established and proven technology for the treatment of animal manure. Although a number of investigators have studied the fate of antibiotics in soil interstitial water [9], and in anaerobic lagoons [10,11], there is very limited information on the fate of OTC during anaerobic digestion of manure. Antibiotic inhibition of anaerobic digestion of pig manure has been reported [7,12], but few studies have been undertaken using calf manure [13]. Moreover, most studies have been conducted by adding antibiotics into the reactor as opposed to using manure from OTC-medicated animals. Fedler and Day [14] observed that the antibiotics themselves may not inhibit bacterial activity but that antibiotic metabolites produced in the gastrointestinal tract of
the animal may. More recently, Halling-Sørensen et al. [1] showed that the degradation products of OTC have less biological activity on sludge bacteria than OTC. However, to best evaluate the fate and effect of antibiotics during anaerobic digestion, manure should be used from animals treated with antibiotics at therapeutic rates. The removal of OTC in manure from medicated calves during manure composting was recently studied [3]. The objective of this study was to determine the fate and effect of OTC during the anaerobic digestion of manure from medicated calves.

2. Materials and methods

2.1. Chemicals

Oxytetracycline (Mₚ = 460, CAS no. 79-57-2), 4-epi-oxytetracycline (Mₚ = 460, CAS no. 35259-39-3), α-apo-oxytetracycline (Mₚ = 442, CAS no. 18695-01-07), β-apo-oxytetracycline (Mₚ = 442, CAS no. 18751-99-0) and demeclocycline hydrochloride (Mₚ = 502, CAS no. 64-73-3) were purchased (97–100% purity) from Acros Organics N.V. (Fair Lawn, NJ). All other reagents used in this study were analytical grade. The water used in the experiments was purified by using reverse osmosis and activated carbon. McIlvaine buffer was prepared by mixing aqueous solutions of 0.1 M citric acid and 0.2 M disodium hydrogen phosphate (62:38, v/v). Methanolic oxalic acid (0.01 M) was prepared by dissolving oxalic acid in methanol.

2.2. Animal medication and sample collection

Five male beef calves, 4–6 months old and ranging from 170 to 240 kg in body mass, were kept in individual pens at the Beltsville Agricultural Research Center (Beltsville, MD) Beef Barn. Pens were scraped clean daily, after which approximately 2 kg of sawdust was scattered on the floor of each pen as bedding material. After a 2-week acclimatization period for the animals, the manure–sawdust mixture from each pen was collected (averaging 15 kg/animal/day), pooled, mixed, and 75 kg of this mixture was stored at 4 °C until later use as the “unmedicated” manure. The calves were then medicated for 5 days at 22 mg/kg body mass per day of OTC (a standard dosage in agricultural practice [15]) by ingestion of the daily ration containing OTC as a feed additive. Feed consisted of a mixture of unmedicated Ncf2 beef creep pellet (9%), sudan silage (17%), corn silage (24%) and medicated Ncf2 beef creep pellet (50%). Medicated grain was given to the animals prior to other constituents in order to insure complete consumption of the OTC dose. Medicated manure–bedding mixtures collected on the fifth day of medication were combined and used in laboratory anaerobic digestion experiments as the “medicated manure”. The OTC content of this manure was determined as described below.

2.3. Anaerobic digestion

Anaerobic digestion experiments were carried out batch-wise in six laboratory digesters (Bellco Biotechnology, NJ, US) each with a working volume of 1225 ml. Medicated manure (containing 62 ± 4 μg/g OTC, 3.4 ± 0.2 μg/g EOTC, 1.8 ± 0.2 μg/g α-Apo-OTC, 0.3 ± 0.1 μg/g β-Apo-OTC, wet weight) from the fifth day of antibiotic treatment was diluted five-fold with tap water to approximately 5% total solids (a level that is representative of digestor influent in commercial farm operations [16]), and 1 l of manure slurry was loaded into each of three digesters. Comparably diluted manure slurry from animals prior to medication was loaded into the three remaining digesters. A 225 ml of effluent from Beltsville Agricultural Research Center dairy manure digester (Beltsville, Maryland) was added to each digester as inoculum. Table 1 shows characteristics of the diluted medicated and unmedicated manure slurries that were loaded into the digesters. After the digesters were filled, the headspaces were flushed with nitrogen gas to remove traces of oxygen. The digesters were stirred continuously to avoid compaction and incubated at a mesophilic temperature (35 ± 1 °C). Manure slurry samples (30 ml) were collected from each digester on days 0, 11, 19, 27 and 64 and analysed for pH, ammonium-N, OTC and OTC metabolites. Manure slurry samples were collected using 50 ml syringe under nitrogen gas. Digesters were stirred continuously in order to collect homogeneous samples during the sample collection. Levels of total solids (TS), total alkalinity, total nitrogen and total phosphorus were determined for the day 0 and 64 samples. Levels of volatile solids (VS) and soluble organic carbon (SOC) were determined for day 0 and 64 samples.

Biogas production was monitored daily by using a water displacement technique [17]. The biogas measurement setup was filled with water containing 40% NaCl. The pH of the water was adjusted to 2.0 by adding HCl to prevent the dissolution of carbon dioxide present in the biogas. Methane content in the biogas was determined weekly by gas chromatography using an HP 5840 instrument, an HP-Plot/Al 203 capillary steel column and a flame ionization detector. The oven, injector and detector temperatures were 100, 150 and 250 °C, respectively. Helium was used as a carrier gas at 60 ml/min.

TS, VS and total alkalinity were determined according to APHA [18]. Total nitrogen and ammonium-N were determined colorimetrically by flow injection analysis (Lachat Instruments, Milwaukee, WI). Samples for SOC analysis were...
Table 1
Characteristics of diluted medicated and unmedicated manure slurries used in anaerobic digesters (mean ± std. error)

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Medicated manure slurry</th>
<th>Unmedicated manure slurry</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.2 ± 0.1</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>Total alkalinity (mg CaCO₃/l)</td>
<td>79800 ± 1010</td>
<td>81700 ± 1600</td>
</tr>
<tr>
<td>Total solids (mg/l)</td>
<td>4980 ± 200</td>
<td>4680 ± 250</td>
</tr>
<tr>
<td>Volatile solids (mg/l)</td>
<td>4410 ± 35</td>
<td>4240 ± 10</td>
</tr>
<tr>
<td>Total nitrogen (mg/l)</td>
<td>1530 ± 40</td>
<td>1670 ± 60</td>
</tr>
<tr>
<td>Ammonium-N (mg/l)</td>
<td>330 ± 30</td>
<td>410 ± 55</td>
</tr>
<tr>
<td>Total phosphorus (mg/l)</td>
<td>360 ± 5</td>
<td>380 ± 7</td>
</tr>
<tr>
<td>Soluble organic carbon (mg/l)</td>
<td>780 ± 50</td>
<td>750 ± 60</td>
</tr>
</tbody>
</table>

subjected to centrifugation (1650 × g, 20 min, 5 °C) and filtration through 0.45 μm membrane filters prior to analysis on a Phoenix 8000 TOC instrument (Tekmar-Dohrmann, Cincinnati, OH) that uses a wet chemical oxidation method (peroxidation oxidation and UV irradiation) followed by measurement of the resulting CO₂ with a nondispersive infrared (NDIR) detector. Total phosphorus content was determined by block digestion and flow injection analysis (Lachat Instruments, Milwaukee, WI).

Significant differences in cumulative biogas production, methane content, VS and SOC reduction during anaerobic digestion due to OTC was determined by t-test using SAS [19]. Significant differences were determined at the p < 0.05 level of significance.

Removal of OTC was assumed to follow first-order kinetics. A rate constant, k, was determined as the slope of the curve calculated by linear regression. The half-life, T½, was then calculated as T½ = ln(2)/k.

2.4. Extraction of OTC and its metabolites

Manure slurry samples were extracted in duplicate for analysis of OTC and its metabolites using the method described by Capone et al. [20]. Briefly, 2 ml samples were extracted three times with 3 ml of 0.1 M Na₂EDTA-McIlvaine buffer by vortexing for 30 s followed by sonication for 3 min in a 100 W sonication bath (Bronson Ultrasoundics, Danbury, CT). After each extraction, the extracts were subjected to centrifugation (500 × g, 5 min, 5 °C), the supernatants were pooled, again subjected to centrifugation, filtered through 2.5 μm Whatman 5 filter paper, and passed through pre-washed Waters Sep-Pak C-18 cartridges. The cartridges were prewashed with 5 ml of methanol followed by 10 ml of 0.1 M Na₂EDTA-McIlvaine buffer. After the extracts were loaded, the cartridges were flushed with 20 ml distilled water, followed by sample elution using 8 ml of 0.01 M methanolic oxalic acid. Eluents were concentrated to approximately 1 ml by evaporation prior to analysis by LC/MS/MS.

2.5. LC/MS/MS analysis

Chromatographic separation was based on a method by Lykkeberg et al. [21]. The LC instrument was a Waters 2690XE (Waters Corp., Milford, MA) separations module with an Xterra MS C₁₈ column (150 mm × 2.1 mm i.d., 5 μm) (Waters Corp., Milford, MA) at 50 °C; the injection volume was 10 μl. A mobile-phase gradient was necessary to separate the compounds: solvent A was methanol–water (95:5, v/v) with formic acid (308 μl/l) added; solvent B was methanol–water (95:5, v/v) with formic acid (308 μl/l) added. The solvents were mixed as follows: 0–6 min 89% A; 6–11 min a linear gradient to 50% A, 11–21 min 50% A, 21–22 min returning to the starting condition. Then, the column was stabilized for 13 min with 89% A. The total run time was 35 min. The flow rate was set at 0.25 ml/min. Atmospheric pressure ionization-tandem mass spectrometry was performed on a benchtop triple quadrupole mass spectrometer (Quattro LC from Micromass Ltd., Manchester, UK) operated in electrospray ionization mode. The source parameters were as follows: capillary voltage was set at 3.5 kV and extractor voltage was set at 3 V, respectively; rf lens at 0.1 V; source and desolvation temperatures were 150 and 450 °C. Liquid nitrogen was used to supply the nebulizer and desolvations gas (flow rates were approximately 80 and 600 l/h, respectively). Argon was used as collision-induced decomposition gas to fragment the parent ions; the typical pressure was 2.6 × 10⁻² mbar. Both high- and low-mass resolutions were set at 12.0 for both quadrupoles. Acquisition was done in the multiple-reaction monitoring mode (MRM) in electrospray positive (ES+). The parent and daughter ions used for compound identification and quantitation are listed in Table 2 along with the optimum cone voltages and collision energies used. Optimization was performed by infusion of the standards from a syringe pump (10 μl/min) mixed with the LC effluent (100% A; 200 μl/min), with high- and low-mass resolution set at 15.0. Detector was a photomultiplier set at 650 V. Analyte concentrations were calculated by the internal standard method using demeclocycline as an internal standard [22]. Peak integration and quantitation were performed automatically using the MassLynx 3.5 software (Waters Corp., Milford, MA).

2.6. Determination of extraction efficiencies for OTC and OTC metabolites

To determine extraction efficiencies, duplicate samples of unmedicated manure slurry were spiked at 1 and 10 mg/l for OTC, 0.5 and 1 mg/l for EOTC, α-Apo-OTC and β-Apo-OTC, incubated 30 min, and extracted as described above. Recovery results shown in Table 3 were calculated as a means of duplicate samples at each concentration. There was no conversion of OTC to its metabolites during the experimental procedure. Recovery of all compounds appeared to be independent of initial concentration. Although recoveries of OTC and EOTC were about 90%, recoveries of α-Apo-OTC and β-Apo-OTC were only 40 and 25%, respectively. Low recoveries of α-Apo-OTC and β-Apo-OTC have been previously reported by Fedeniuk et al. [23] in studies using porcine tissue.

Table 2
Parent and daughter ions used for quantitation of OTC and its metabolites and MS parameters used to produce them

<table>
<thead>
<tr>
<th>Compound</th>
<th>Parent ion (Da)</th>
<th>Daughter ion (Da)</th>
<th>Retention time (min)</th>
<th>Cone (V)</th>
<th>Collision (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EOTC</td>
<td>461</td>
<td>426</td>
<td>4.5</td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>OTC</td>
<td>461</td>
<td>426</td>
<td>7.2</td>
<td>23</td>
<td>18</td>
</tr>
<tr>
<td>α-Apo-OTC</td>
<td>443</td>
<td>426</td>
<td>13.6</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>β-Apo-OTC</td>
<td>443</td>
<td>426</td>
<td>19.6</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>Demeclocycline</td>
<td>465</td>
<td>448</td>
<td>9.8</td>
<td>26</td>
<td>18</td>
</tr>
</tbody>
</table>
3. Results and discussion

3.1. The fate OTC during anaerobic digestion

Anaerobic digesters were incubated for 64 days at 35 °C using five-fold diluted manure collected from calves prior to and after OTC medication. The levels of OTC and its metabolites during anaerobic digestion are shown in Fig. 2. OTC levels decreased from 9.8 ± 0.1 to 6.8 ± 0.2 mg/l within 11 days (a 30% reduction), then remained relatively constant until the fourth week of digestion after which OTC concentrations decreased to 4.0 ± 0.1 by day 64 (Fig. 2a). Overall, a 59% removal of OTC was achieved during 64 days of anaerobic digestion, yielding a calculated OTC half-life value of 56 days. Winckler and Grafe reported a 55–57 day tetracycline half-life value using 20 and 100 mg/ml spiked concentrations in pig slurry incubated at 38 °C [24]. They also reported a 105 day half-life value using 20 mg/ml spiked concentration in outdoor experiments. In contrast, Kühne et al. [25] reported half-life values of only 4.5 and 9 days at ambient temperature for tetracycline in aerated and non-aerated pig manure (containing 200 μg/ml tetracycline), respectively.

EOTC levels increased gradually from 0.55 ± 0.03 mg/l at the start of experiment to 1.3 ± 0.1 mg/l on day 27 and then decreased to 0.84 ± 0.04 mg/l on day 64 (Fig. 2b). α-Apo-OTC concentrations declined from 0.30 ± 0.03 to 0.14 ± 0.02 mg/l over the 64 day incubation period, but there was no significant change of β-Apo-OTC levels over this period (Fig. 2c and d). Both apo-OTCs were found at a relatively low level (about 3%) relative to OTC. These metabolites were present in the manure from medicated calves, and their levels did not increase during the anaerobic digestion process.

3.2. Effect of OTC and its metabolites on anaerobic digestion

3.2.1. Biogas production

Cumulative biogas production values from digesters containing medicated and unmedicated manure slurries are shown in Fig. 3. Biogas production was observed from all digesters with no lag phase and all digesters reached peak biogas production by the fifth day of incubation. After 64 days, the mean cumulative biogas production values for digesters containing medicated and unmedicated manure were 13,800 ± 110 and 18,900 ± 650 ml, respectively. The methane productivity of medicated and unmedicated manure in terms of volatile solids were 184 ± 1 and 256 ± 9 l/kg/VS, respectively. These results are comparable with the methane productivity values (220–270 l/kg/VS) listed from various sources for beef manure [26]. Cumulative biogas production was 27% lower (p < 0.05) from digesters containing manure from medicated calves relative to that from digesters.
containing unmedicated manure. This result is comparable to those of a previous study in which 32, 40 and 49% biogas production reductions were observed during batch mesophilic anaerobic digestion of cow manure containing OTC spiked levels of 12.5, 37.5 and 75 mg/l, respectively [13]. In contrast, Lallai et al. [12] did not observe any reduction of methane production during batch mesophilic anaerobic digestion of pig manure using 125 and 250 mg/l OTC. Sankvist et al. [27] investigated the effect of OTC on the anaerobic digestion of pig manure slurry for batch and semi-continuous systems in 3.5 l fermentors operated at mesophilic (37 °C) and thermophilic (55 °C) temperatures. They reported that in semi-continuous flow thermophilic fermentors with a hydraulic retention time (HRT) of 5–7 days, methane production was reduced by 50% after 6 consecutive days of adding OTC at a rate of 100 mg/l to the pig manure slurry. They also showed 35% (in 15 days) and 55% (in 25 days) inhibition of biogas production for thermophilic and mesophilic batch experiments, respectively. Fedler and Day [14] reported that manure (2 mg/l chlortetracycline concentration) from medicated pigs reduced methane production by about 20% in 3 l semi-continuous flow reactors at 35 °C. However, when chlortetracycline was added directly into the fermentors, there was no reduction in methane production. They speculated that the inhibition was not due to antibiotic itself, but rather due to metabolites produced as a product of the swine gastrointestinal tract. Sanz et al. [28] showed 20, 50 and 80% inhibition of methane production during batch mesophilic anaerobic digestion of artificially prepared volatile fatty acids (acetate, propionate and butyrate) at spiked levels of 5, 40 and 152 mg/l chlortetracycline concentrations.

3.2.2. Process stability

The presence of OTC in manure from medicated calves showed no effect on pH during digestion. Initial pH values for digesters containing medicated and unmedicated manure were 7.2 and 7.1, respectively (Fig. 4a). pH values increased in both medicated and unmedicated manure digesters during the first 3 weeks of digestion and then remained between 7.6 and 7.9. An optimal pH range of 6.7–7.4 has been reported for most methanogenic bacteria and the rate of methanogenesis may decrease if the pH is <6.3 or >7.8 [29].

The presence of OTC in manure from medicated calves also showed no significant effect on biogas composition or on reductions in volatile solids and soluble organic carbon. The average percentages of methane in the biogas produced from medicated and unmedicated manure digesters were 60/3 and 61/3%, respectively (p > 0.05). The VS reductions were 57 ± 3% for medicated manure digesters and 59 ± 4% for control unmedicated manure digesters (p > 0.05). The SOC reductions were 63 ± 4% for medicated manure digesters and 61 ± 6% for unmedicated manure digesters (p > 0.05). Similar results have been reported previously in experiments using pig manure containing chlortetracycline [14,30]. Fedler and Day [14] did not find any difference of methane content and VS reduction at 20% methane production inhibition by 2 mg/l chlortetracycline concentration. Varel and Hashimoto [30] found that although chlortetracycline reduced the methane production rate by approximately 20%, the methane content was not affected.

Ammonium can be toxic to methanogenic archea during anaerobic digestion. Therefore, digesters were monitored for ammonium-N in order to determine whether reduced biogas production might be caused by high levels of ammonium-N (Fig. 4b). Ammonia-N levels increased to maximum levels of 482 ± 39 and 512 ± 49 mg/l for medicated and unmedicated
manure digesters, respectively. These levels are well below the threshold concentration of 1700 mg/l that would cause inhibition [31].

Anaerobic digestion of manures can result in energy generation that can supplement or replace purchased energy from local suppliers. Some farmers generate excess energy that is then sold to local suppliers. Our data show that OTC and probably other antibiotics reduce biogas output and farmers should be aware of this effect when medicating animals whose manure is then placed into anaerobic digesters. The results can be increased energy costs and/or lost revenue. Farmers should be advised of the effects of OTC and adjust medication or segregation of manure especially within the first week of treatment. Other manure treatments are more effective in reducing OTC. We have recently demonstrated that composting can reduce levels of extractable OTC in manure (a half-life of 3 days compared to the 56 day value reported here for anaerobic digestion [3]). This composted manure from medicated animals could then be land applied. Even though OTC does not affect process stability, biogas reduction should be considered when manure from medicated animals is fed to digesters.

4. Conclusions

Approximately 60% removal of OTC was achieved in 64 days by anaerobic digestion at 35 °C yielding a calculated value of 56 days of OTC of 65 days. However, our experiments do not resolve whether the reduction of OTC is caused by degradation, mineralization or binding of OTC to the organic matrix. In addition (and in common with numerous other studies focused on the degradation of xenobiotics in organic matrices), these experiments do not separate the relative contributions of individual factors that occur during digestion (elevated temperatures, high biological activity, biologically transformed organic material) that affect levels of extractable OTC nor the interactions between these factors.

OTC reduced biogas production by 27% during these batch experiments. However, OTC did not have observable negative effects on process stability as there were no significant differences in biogas methane content or in reductions of volatile solids or soluble organic carbon.

Acknowledgments

The authors are grateful to veterinarian Dr. William Hare and the Beltsville Agricultural Research Center Beef Barn animal caretaker staff for conducting the animal feeding study, Greg McCarty and Swati Mookherji for their assistance with GC measurements.

References


