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Assessing the influence of pig slurry pH on the degradation of selected antibiotic compounds

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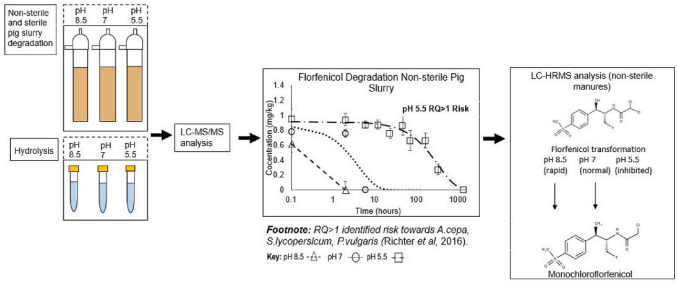
HIGHLIGHTS

- The effect of pig slurry pH on antibiotic degradation was compound specific.
- Abiotic and biotic processes contributed to this phenomenon.
- Differing route and rate of transformation products were observed.
- Manure degradation trials should employ multiple manures of differing properties.

GRAPHICAL ABSTRACT



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ABSTRACT

Veterinary medicines are routinely used in animal husbandry and the environment may consequently be exposed to them via manure applications. This presents potential environmental and societal risks such as toxicological effects to aquatic/terrestrial organisms and the spread of antimicrobial resistance. Regulatory studies that assess the degradability of veterinary antibiotics during manure storage currently permit the use of just one manure per animal type although we speculate that heterogenic properties such as pH could be driving significant variability within degradation rates. To bridge this knowledge gap and assess degradation variability with pH, laboratory degradation studies were performed on a broad range of antibiotics (ceftiofur, florfenicol, oxytetracycline, sulfamethoxazole and tylosin) at three different environmentally relevant pH levels (5.5, 7, and 8.5). The effect of pig slurry pH on degradation rates was found to be significant and compound specific. Usually, acidic slurries were found to inhibit degradation when compared to neutral pH, for florfenicol, tylosin, and ceftiofur; the associated changes in DT_{50} (half-life) values were 2–209 h, 35.28–234 h, and 0.98–2.13 h, respectively. In some circumstances alkaline slurries were observed to enhance the degradation rate when compared to those for neutral pH, for tylosin, the respective changes in DT_{50} values were from 3.52 to 35.28 h. Comparatively, the degradation of sulfamethoxazole was enhanced by acidic conditions compared to neutral (DT_{50} 20.6–31.6 h).

Abbreviations: Liquid Chromatography Mass Spectrometry, (LC-MS); Liquid Chromatography High Resolution Mass Spectrometry, (LC-HRMS); half-life, (DT_{50}); below detectable limits, (bdl); ceftiofur, (CFT); florfenicol, (FLO); tylosin, (TYL); sulfamethoxazole, (SMX); oxytetracycline, (OTC).

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Tentative identification of unknown transformation products (TPs) was achieved for sulfamethoxazole and florfenicol for the first time in pig slurries. These results reveal the importance of considering slurry pH when assessing the degradation of antibiotic compounds, which has implications for the acidification of manures and the environmental risk assessment for veterinary medicines.

Environmental relevance and significance: Given the significant effect of pig slurry pH on degradation rates, manure degradation studies need to be harmonised and standardized, taking into account the influence of pH.

1. Introduction

Livestock are routinely administered veterinary medicines to improve or protect animal health. As a consequence of poor absorption within the gastrointestinal tract of the animal, high percentages of veterinary medicines are often excreted as the parent compound or their metabolites (Thiele-Bruhn, 2003). Excretion rates of pharmaceuticals depend on specific pharmacodynamic and pharmacokinetic processes/properties and vary between 10 and 90% of the administered dose (Hirsch et al., 1999; Montforts et al., 1999; Sukul et al., 2009). The environment is exposed to veterinary medicines via manure applications to land or directly via excretion from animals reared on pasture (Kemper, 2008). The usage of animal manures as a fertilizer is commonplace within agriculture, primarily due to its excellent nutrient content, but also because application to land offers a suitable route for waste disposal (Potter et al., 2010; Lee, 2010; Bogaard et al., 2013; Salgado et al., 2019).

Veterinary medicines that are present within the terrestrial environment can migrate to the aquatic environment via, runoff to surface water or leaching into groundwater (Boxall et al., 2004; Kay et al., 2005; Kreuzig et al., 2005; Li et al., 2018). Common on-farm practice is to store animal slurries, during which veterinary antibiotics are subject to various degradation and dissipation processes such as microbial mineralization, adsorption, hydrolysis, and chemical transformation (Lamshöft et al., 2010). Typically, pig slurry is stored on average for 53 days within the EU, during which time veterinary medicines are subject to varying rates of degradation (EMA, 2016).

Within the environmental risk assessment for veterinary medicines slurry incubation studies are employed to assess the degradability of veterinary medicines during this timeframe. This provides a more accurate estimate of concentrations exposed to land following manure application (EMA, 2011). Presently there is a lot of uncertainty regarding veterinary medicine degradation within manures due to differences in manure properties; despite the fact that a thorough understanding is critical for harmonisation of manure degradation studies (EMA, 2011; VICH, 2000; VICH, 2003). Variation exists between manure degradation studies, for example Blackwell et al. (2005) reported a DT_{50} of 79 d for oxytetracycline within pig slurries whereas Wang et al. (2015) calculated a range of 9.05–9.65 d. When regulatory soil degradation studies are performed (e.g., OECD 307), the use of four soil types is usually required due to the known influence of soil properties on the degradation rate and route of organic compounds (OECD, 2002; Junker et al., 2020). Soil matrix properties can affect sorption, hydrolysis, microbial mineralization as well as the ionic strength and speciation of contaminants (pKa) via changes in pH (Sassman and Lee, 2005; ter Laak et al., 2006; Chatterjee et al., 2013; Jechalke et al., 2014; Mitchell et al., 2014; Junker et al., 2020). Most regulatory manure degradation studies consider one animal manure per animal type, but we hypothesise varied manure properties such as pH to be of equal or greater importance to those of soils.

The pH of pig slurries is heterogenic and is related to manure management (acidification), animal feed, animal age, water, temperature, redox potential, as well as the aging process. Through these mechanisms and the degradation of organic matter, different ratios of ammonia, ammonium and volatile fatty acids (fulvic and humic acids) form thereby altering slurry pH (Paul and Beauchamp, 1989; M⊘ ller et al., 2004; Page et al., 2014; Joubin, 2018). Animal feed is well known to directly influence slurry pH and higher protein diets have been identified to increase pH whilst carbohydrates decrease the pH (Canh et al., 1998a, 1998b). Typically pig slurry is expected to be neutral in pH, however, a wide range of pig slurry pHs are documented within the literature, for example Cooper and Cornforth (1978) noted a range of 6.5–7.5 and Weinfurtner (2011) observed pH values of 13 pig slurries in Germany to be in the range of 5.55–9.14. Within the literature there are publications reporting extremely acidic pig slurries (e.g., 4.8, 5.11) (Choudhary et al., 1996; Thiele-Bruhn, 2003a; Martinez-Suller et al., 2008; Wang et al., 2015; Shan et al., 2018), as well as alkaline pig slurries (e.g., pH 9.14, 8.92, and 8.11). (Sommer and Husted, 1995; Hu et al., 2011; Mroz et al., 2000).

Despite documentation of wide-ranging pH values in pig slurries and the potential impacts on veterinary medicine degradation, studies of the effect of pH are lacking (Ratasuk et al., 2012; Ali et al., 2013). Therefore, the aim of this research was to address this significant knowledge gap and quantify the effect of various slurry pHs on the degradation of a wide range of veterinary antibiotics, including their transformation products. This work has the potential to contribute towards a more robust environmental risk assessment for veterinary medicines.

2. Methodology

2.1. Chemical compounds and stock preperation

All chemicals and solvents were of the highest available purity. Methanol, orthophosphoric acid, H_2SO_4 (86%), NaOH (98%), Na_2EDTA, citric acid and di-sodium hydrogen orthophosphate were purchased from Fischer Scientific (UK). Analytical grade antibiotics were used (94–98%); oxytetracycline (OTC), ceftiofur (CFT) and tylosin tartrate (TYL) were purchased from Scientific Laboratory Supplies SLS (UK), whilst florfenicol (FLO) and sulfamethoxazole (SMX) were purchased from VWR (UK). The influence of pH on degradation rates will likely be compound specific, therefore, a suite of veterinary antibiotics with differing modes of action were selected to encompass a wide range of physical-chemical properties (Table .1) (i.e., pKa, Log K_{ow}, K_d, and molecular weight). Stock solutions were prepared in methanol and fresh matrix matched calibration standards were made following the extraction of pig slurries and aqueous samples. 0.1 M Na_2 McIlvaine buffer pH 4 (50:50) was prepared by mixing 307.25 mL of 0.1 M citric acid, 192.75 mL 0.2 M Na_2HPO_4 and 500 mL of 0.1 M Na_2EDTA. Kolthoff and Vleeschouwer buffers were prepared at varying pHs via OECD 111 (OECD, 2004). To obtain pH 5.5 buffer, 68 mL of 0.1 M NaOH and 50 mL 0.1 M citric acid were combined, whilst to obtain a pH of 7, 68 mL of 0.1 M NaOH, 50 mL boric acid and 0.37 g KCl were mixed.

2.2. Pig slurry sampling, conditioning and characteristics

10 kg of fresh manure was sampled from a pig farm in Welburn, York (UK), the sample was obtained from pigs that were 12 weeks old. The pigs had received sulfadiazine and trimethoprim during their first 6 weeks of life, which is standard husbandry practice when pigs are within the gestation, farrowing and weaning periods (Filippitzi et al., 2014; Lekagul et al., 2019). However, none of the study compounds had been administered prior to collection. Pig manure was stored under anaerobic

conditions at room temperature for no longer than two weeks prior to usage within the study. The manure was homogenised before the moisture content was derived and then adjusted into a slurry using tap water to attain a dry matter content of 5% (d/w). (EMA, 2011).

To characterise the dissolved fraction of pig slurries, the sample was centrifuged at 3250 rpm (2 h) and decanted, the supernatant was then filtered using G/F and 1 PS Whatman papers, followed by a 20 μm G/F and 0.45 μm nylon syringe filter. Analysis of the sample was achieved using an Analytik Jena Multi NC2100 (carbon), Autoanalyzer (nutrients) and ICP-OES (iCAP 7400 Radical) for metals, please refer to Supplementary Information (SI 1.0) for further details. The pig slurry properties were as follows, pH 7.2 ± 0.5, NH_4–N 22.72 mg/L NO_2–N 0.2 ng/L, PO_4–N 24 ng/L, OC 174 ± 6.6 mg/L, inorganic carbon 1087.9 ± 14.3 mg/L, Co 0.05 mg/L, Zn 0.313 mg/L, Cd 0.003 mg/L, Cu 0.032 mg/L and Pb 0.006 mg/L. These properties were found to be comparable to previously published pig slurry characteristics (Weinfurtner, 2011; Sommer et al., 2015).

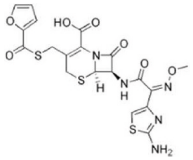
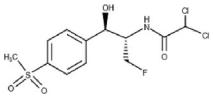
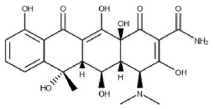
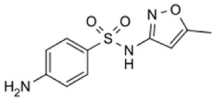
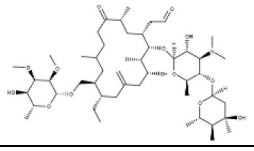
2.3. Biodegradation experiment

Due to a mixture of antibiotics being tested the slurry was dosed with a mixture of five compounds at 20% of the Predicted Environmental Concentration (PEC) which were calculated using the Spaepen (Spaepen et al., 1997) model (SI Table .1). An exception of this was ceftiofur which was dosed at 40% due to the very low PEC and poor analytical sensitivity. The dosage concentrations were 3 mg/kg, 1 mg/kg, 5 mg/kg, 3 mg/kg, and 3.3 mg/kg for CFT, FLO, OTC, SMX and TYL respectively. The degradation of the five antibiotic compounds was performed at three pig slurry pHs; alkaline (pH 8.5), neutral (pH 7) and acidic (pH 5.5). The pH of the pig slurry was controlled using either 3 M H_2SO_4 or 3 M NaOH; daily checks were conducted, and the required adjustment frequencies varied depending on the pH treatment. The volume of acid/base that was added were recorded and the differences in the moisture contents between the treatments was corrected. The incubation vessels facilitated continuous pH and ORP monitoring and subsampling via permanently installed probes and a detachable lid (SI Fig. 1). The vessel lids contained an inlet and outlet facilitating the purging of the headspace with nitrogen, this helped maintain anaerobic conditions with redox potentials values ranging from −250 mV to −400 mV (EMA, 2011). pH conditions were acclimated for ten days prior to dosing to allow the microbial populations to equilibrate to the manipulated pHs (Fangueiro et al., 2015a). Dosing of the pig slurries was achieved using a mixed stock solution that was completely dissolved within methanol; the percentage of methanol to pig slurry was ≤2% to avoid inhibition of the acclimated microbial populations (EMA, 2011). Subsequently sub-samples of the slurries were taken using a serological pipette and the concentrations of antibiotics were measured at 0 h, 2 h, 6 h, 12 h, 24 h, 48 h, 3 d, 7 d, 14 d, 28 d and 56 d. During the incubation the vessels were kept in the dark at 23 ± 0.5 °C, both temperature and light were controlled during this timeframe using a tinytag datalogger (temperature) and a room with the exclusion of light.

2.4. Abiotic degradation experiments

In order to assess abiotic processes, sterile slurry degradation and hydrolysis experiments were performed. The sterile manure experiment was performed with the same conditions (pH, temperature and light) as the non-sterile experiment (except for sterilsation). The slurries were moisture corrected and autoclaved at 120 °C for 30 min at 0.206 bar. Formaldehyde was used to maintain sterility during the studies duration; during the acclimation period the starting concentration of formaldehyde was 0.3%, subsequently, 1.42 mL of formaldehyde was added weekly. Sterility of the test system was checked weekly using nutrient broth and swab checks and, if required, further confirmations were achieved using agar plates. Sterile swabs were used to sample the slurry and the nutrient broths were incubated at 30 °C for 12 h. Visual comparisons for turbidity (bacterial growth) were made between the respective controls and the sample. The hydrolysis analysis was also conducted under the same experimental conditions as both manure degradation studies. For hydrolysis, Kolthoff and Vleeschouwer buffers were set up at varying pHs as per the OECD 111 guideline (OECD, 2004). Buffers containing the antibiotics were stored in 10 mL glass culture

Table 1
Physiochemical properties of the selected veterinary antibiotics and their respective chemical structures.

Antibiotic, Group and Molecular Weight (g/mol)	Chemical Structure	Log K_{ow}	pKa	K_d (mL/g)	K_{oc} (mL/g)
Ceftiofur (Cephalosporin) 523.56 (Yalkowsky and He, 2003)		1.6 (US EPA, 2006)	2.68 (Ribeiro et al., 2018)	14.5–31.72 (An et al., 2021)	755.60–944.66 (An et al., 2021)
Florfenicol (amphenicol) 358.21 (FDA, 2013)		0.37 (FDA, 2013)	9.03 (FDA, 2013)	0.07–0.59 (FDA, 2013)	10 - 27 (FDA, 2013)
Oxytetracycline (Tetracycline) 460.44 (Yalkowsky and He, 2003)		−1.22 (ter Laak et al., 2006)	3.27, 7.32, 9.11 (Stephens et al., 1956)	540 - 1026 (Rabølle and Spliid, 2000)	27792–93317 (Rabølle and Spliid, 2000)
Sulfamethoxazole (Sulphonamide) 253.27 (Yalkowsky and He, 2003)		0.89 (Hansch et al., 1995)	1.44, 5.7 (Srinivasan et al., 2010)	1.13–2.41 (Hu et al., 2019)	12.36–23.99 (Hu et al., 2019)
Tylosin (Macrolide) 916.1 (Yalkowsky and He, 2003)		2.5 (ter Laak et al., 2006)	7.1 (ter Laak et al., 2006)	3 - 156 (ter Laak et al., 2006)	136.36–5032.26 (ter Laak et al., 2006)

Adsorption/desorption details (An et al., 2021): – *Range of a sandy loam, loam and clay soils* (FDA, 2013) – *Range of four standardized soils* (ter Laak et al., 2006), – *Range of loamy sand to clay loam* (Rabølle and Spliid, 2000), – *Range of soils from sandy soil to a loamy sand* (Hu et al., 2019), –*Range of a sandy clay loam and a sandy loam.*

tubes and destructively sampled and filtered using a 0.2 μm nylon syringe filter prior to analysis.

2.5. Extraction and LC-MS/MS analysis

25 ± 0.5 g of slurry was retrieved from each vessel and 50 mL of extraction solvent added (0.1 M Na_2EDTA-McIlvaine buffer pH 4). Samples were shaken at 250 rpm for 20 min using a rotary bed shaker, then centrifuged at 3200 rpm (4 °C) for 20 min; the supernatant was collected, and the extraction repeated. Supernatants were filtered separately to 0.2 μm using a nylon syringeless filter and samples were then stored at −20 °C prior to analysis. The pig slurry extraction procedure was validated at three concentrations: 1%, 10% and 100% of the application concentration. A range of recoveries were achieved (SI Table .2), however both FLO and CFT were not validated for 1% of the PEC due to sensitivity issues accompanied by low dosage concentrations. At 100% all compounds except CFT met the 70% extraction criteria required under the guidance document SANTE (SANTE, 2021). Hydrolysis samples were validated to 1% and 120% of the dose and recoveries ranged from 86 to 115% for all of the assessed compounds.

Detection and quantification of veterinary antibiotics was achieved using a singular method on a SCIEX Triple Quad 5500+ LC-MS/MS which utilized High Performance Liquid Chromatography (HPLC) reversed phase methodology. A Phenomenex Kinetics XB-C18 2.6 μm LC column (50 × 2.1 mm) was used and the mobile phases consisted of 0.1% formic acid in methanol and 0.1% formic acid (aqueous). A 15 μl injection volume and a flow rate of 0.4 mL/m was utilized, and the duration of the method was 11 min with the following gradient, 0 min (0% B), 3 min (90% B), 8 min (90% B), 8.1 min (10% B), 11 min (0% B). Analyst 1.6 software (SCIEX) was used to process and quantitate the samples. The LOD's of the assessed analytes in pig slurries were found to range from 0.000025 to 0.05 mg/kg, whilst the LOQ's ranged from 0.006 to 0.073 mg/kg (see SI Table.3 for specific LODs/LOQs and MS methodology). Quality controls were utilized within the analysis of veterinary medicines to ensure precision and accuracy during the data acquisition (please see SI section 2.0 for further details).

2.6. Liquid chromatography-high resolution mass spectrometry (LC-HRMS)

A Thermo Scientific Exactive Orbitrap LC-HRMS system was used to analyse samples from the 2 h, 6 h, 24 h, 48 h and 14 d timepoints. An ACE 3Q aqueous (150 mm by 3 mm, 3 μm) LC column was used, the mobile phases consisted of methanol: acetonitrile (50:50) with 0.1% formic acid (mobile phase A) and H_2O with 0.1% formic acid in methanol (mobile phase B). The gradient was 37 min in total and utilized reversed phase chromatography, the details were as follows, 0–5 min (0% B), 5–20 min (100% B), 20–30 min (100% B), 30–35 min (0% B) and 37 min (0% B). A 25 μl injection volume, 0.4 mL flow rate and column oven temperature of 40 °C was utilized. Both positive and negative ionization electron spray conditions were assessed in separate runs. Calibrations were performed prior to ensure the instruments mass accuracy (±5 ppm). Using Thermo Fisher software Xcalibur 2.2 (SP1) qualitative processing techniques were utilized to identify potential TP peaks. An in-house database of 66 possible phase 1 TPs was used and the processing mass tolerance windows were set at ±5 ppm and ±10 ppm for the positive and negative conditions respectively. Occurrence of a specific TP was confirmed and tentatively identified through respective comparisons to the controls (i.e., presence vs absence). Peak areas were collated, and comparisons were made between pH treatments; due to peak area (Cps) being used, the comparisons made are relative between samples and do not reflect absolute concentration. Unfortunately, standards for all TPs were not available to determine absolute concentrations or confirm identities. Nevertheless, the observed trends, in relative abundance, reveal findings with regards to the TP rate or route assessments.

Table 2
Degradation rates (DT_{50}) of veterinary antibiotics under different pH conditions and treatments, non-sterile slurry, sterile slurry and aqueous buffer (hydrolysis).

Compound	Matrix	pH 8.5	pH 7	pH 5.5
Ceftiofur	*Pig slurry*	0.59 h	0.99 h	2.13 h
	Sterile pig slurry	6.5 d	6.5 d	2.33 d
	Aqueous buffer	19 d	49.16 d	75 d
Florfenicol	*Pig slurry*	<2 h	<2 h	8.7 d
	Sterile pig slurry	30.95 d	79.58 d	45.83 d
	Aqueous buffer	>416 d	>416 d	>416 d
Oxytetracycline	*Pig slurry*	78 d	90 d	29.04 d
	Sterile pig slurry	38.8 h	30.7 h	17 h
	Aqueous buffer	17.8 d	9.4 d	11 d
Sulfamethoxazole	*Pig slurry*	122.8 h	31.6 h	20.6 h
	Sterile pig slurry	32.5 d	23.7 d	5.04 d
	Aqueous buffer	>416 d	>416 d	80.41 d
Tylosin	*Pig slurry*	3.52 h	35.28 h	234 h
	Sterile pig slurry	165 d	245 d	675 d
	Aqueous buffer	>416 d	>416 d	114 d

Footnote – For compounds and assessments which were inherently persistent a DT_{50} value of >416 d has been selected. A consequence of not doing so would result in trends and forced modelled predictions, the stability past this point is unknown.

2.7. Degradation kinetics and statistical analyses

Modelling software (CAKE v3.3) was used to derive the kinetic profile; the same kinetic models were used throughout all pH treatments facilitating statistical comparisons. The most appropriate fit for all three pHs was selected, ensuring the residual plots had a chi-squared <15%. In order to obtain the degradation kinetics of the identified TPs, peak areas were plotted against a normalized time (i.e., where degradation initiated). The degradation software was used to derive the DT_{50} values using Single First Order (SFO) and First Order Multi Compartment (FOMC) models (Eq 1 + 2).Where: *k* is the degradation rate constant, α is alpha and β is beta.

$$DT_{50}\ (SFO) = \ln/k \qquad \text{Equation 1}$$

$$DT_{50}\ (FOMC) = \beta\left(2^{\frac{1}{\alpha}} - 1\right) \qquad \text{Equation 2}$$

Statistical analyses were completed using Minitab 18. An analysis of variance (two-way) was conducted on the sample data to statistically compare between the three pH treatments over time for each analyte (concentration = time*pH), a three-way ANOVA was conducted for the analyses of time*pH*treatment. Statistical analysis was conducted using five replicates (n = 5) and statistical significance was reported at the 95% confidence interval (p value = ≤ 0.05). Tukey post hoc comparisons were undertaken on the analyses of variance to distinguish comparisons between pHs and treatments.

3. Results

3.1. Degradation within pig slurries at differen't pHs

The degradation data was best fit to SFO kinetics for CFT, OTC, FLO and SMX, whereas TYL followed a bi-phasic degradation pattern, therefore the FOMC was the best suited model (Fig. 1). Generally, the persistence of each compound under neutral slurry conditions followed this hierarchy: FLO < CFT < TYL < SMX < OTC**.** OTC was persistent throughout the experiment and the study's duration (56 d) was not adequate to fully assess the degradability of this compound within the different slurry pHs.

An ANOVA (two-way) revealed the significant effect of non-sterile pig slurry pH on the degradation of FLO ($p \leq 0.05$), TYL ($p \leq 0.05$), SMX ($p \leq 0.05$) and to a lesser extent CFT ($p \leq 0.05$) (Fig. 1 and

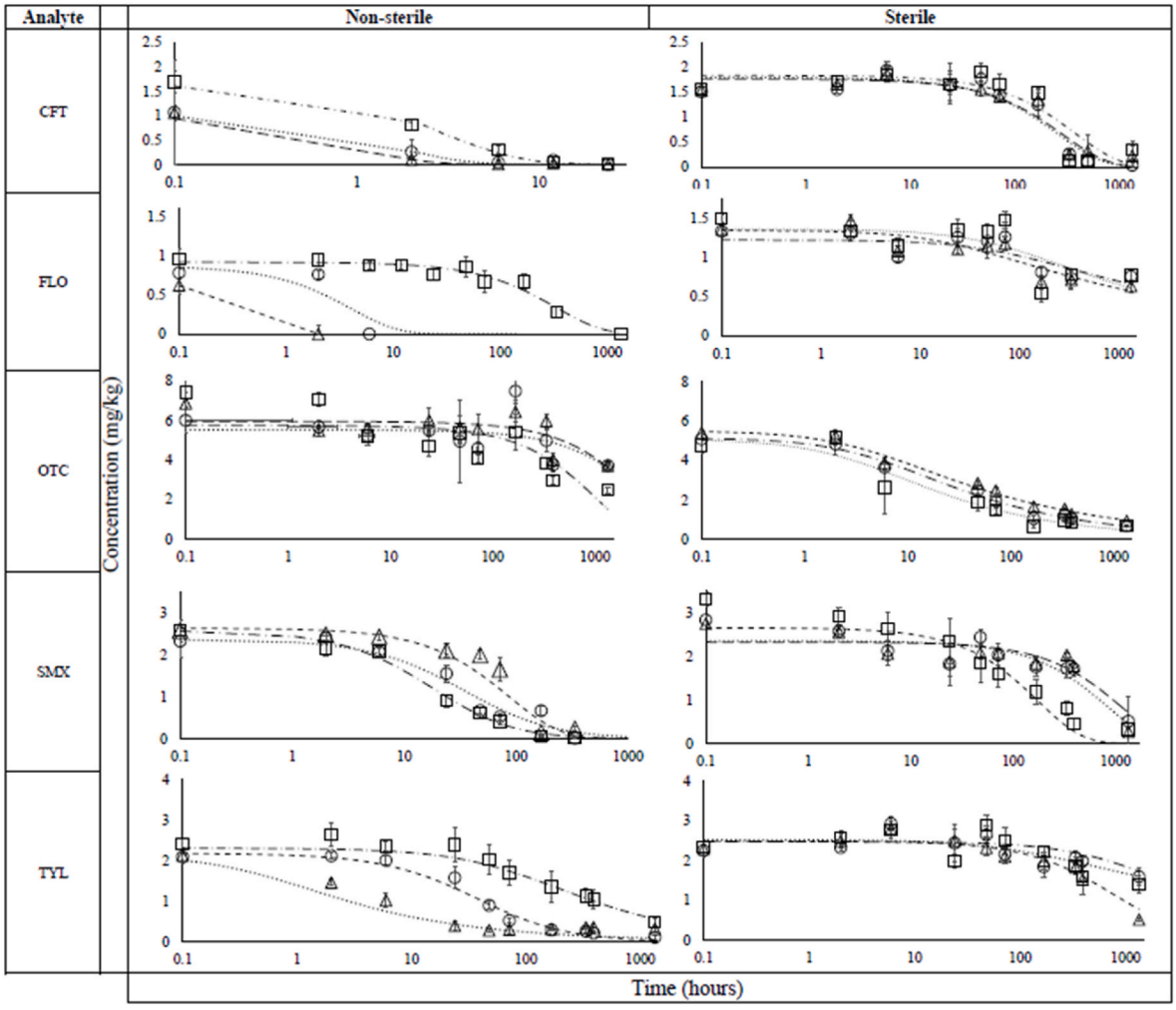


Fig. 1. Degradation of selected veterinary antibiotics at varying pHs over time within non-sterile pig slurry and sterile pig slurry. The x-axis is presented logarithmically, the error bars represent the standard deviation of five replicates.

Table .2). Within acidic pig slurries the degradation rates of FLO, CFT, and TYL were significantly inhibited over that of the neutral treatment ($p \leq 0.05$), whilst alkaline conditions were found to promote degradation ($p \leq 0.05$) (Fig. 1). FLO degradation within acidic slurries exhibited a lag phase of 72 h and the degradation rate was reduced by a factor of over 100 in comparison to that of neutral (k 0.00229 < 0.2451) ($p \leq 0.05$). This resulted in a 100-fold increase in DT_{50} in comparison to neutral/alkaline conditions (2 h–8.7 d) (Table .2 and Fig. 1). Similarly, TYL degradation within acidic slurries was inhibited and also experienced a lag phase, resulting in a six-fold increase in the DT_{50} value when compared to pH 7 (1.47–9.75 d) ($p \leq 0.05$). Interestingly, under alkaline conditions TYL exhibited a ten-fold increase in degradation rate over that of neutral (3.52–35.26 h) (Fig. 1 and Table .2) ($p \leq 0.05$). Comparatively, acidic conditions promoted the degradation rate constant for SMX over that of neutral (k 0.3976 < 0.4458) ($p \leq 0.05$), whilst alkaline pH was found to significantly hinder the degradation (Fig. 1), thus driving differences within the calculated DT_{50} values, these were 20.6 h at pH 5.5, 31.6 h for pH 7 and 122.8 h at pH 8.5 (Table .2).

3.2. Abiotic degradation in sterile slurry

Of the selected antibiotics, FLO, CFT, TYL and SMX degradation rates in sterile slurry were found to be significantly reduced during the analysis of variance (three-way), when they were compared to the non-sterile treatment ($p \leq 0.05$) (Fig. 1 and Table .2). However, OTC under sterile conditions was observed to degrade faster over that of non-sterile; a 70-fold increase in degradation was noted at a pH of 7 between non-sterile and sterile treatments (Fig. 1 and Table .2).

Degradation in sterile slurry treatments was not significantly affected by the slurry pH for FLO and CFT. However, the analysis of variance (two-way) revealed that pH treatment drove differences for SMX, and to a lesser extent TYL. Faster dissipation was exhibited for SMX within sterile acidic conditions (DT50 5.04 d) compared to that of neutral (DT_{50} 23.7 d) ($p \leq 0.05$) (Table .2). Increased elimination of TYL was observed under sterile alkaline conditions at 56 d. Although this trend was deemed insignificant, it revealed some degree of difference to that of neutral ($p = 0.094$); a 700-fold increase in β rate constant was observed (Table .2).

3.3. Hydrolysis

A three-way ANOVA demonstrated that the rates of degradation for CFT, FLO, SMX and TYL within the hydrolysis experiment were significantly lower than in the slurry system ($p \leq 0.05$) (Table .2). For example, at pH 7, FLO persisted for 56 d and the concentration did not change. Comparatively, OTC was found to be hydrolytically unstable which resulted in a DT_{50} of 9.3 h at pH 7. The influence of pH within buffers was very minor or had no significant effect on the elimination of FLO, OTC and TYL. Differences in pH and hydrolytic degradation rates were observed for both CFT and SMX, CFT degradation was increased in

alkaline buffers whilst SMX was promoted under acidic conditions ($p \leq 0.05$) (Table .2 and SI Fig. 2).

3.4. Identification of transformation products within NON-STERILE pig slurry

A large spectrum of known and unknown TPs was tentatively identified for all of the study compounds in the non-sterile pig slurry treatments (SI Table.4). Monochloroflorfenicol (MCF) was tentatively identified within pig slurries, this TP is formed via the loss of a chlorine adduct and the exact mass was 323.03942 *m/z*. Moreover, TPs of FLO were detected for the first time in pig slurries, referred to as FLO_M_338 and FLO_M_271; the structural changes proposed for these TPs were the addition of H_2 +O (338.98990 *m/z*) and the loss of -Cl_2, –O (271.06784 *m/z*). OTC degraded into metabolites referred to as APO-OTC, A-APO and B-APO OTC which are isomers with the same mass (442.13760 *m/z*) and could not be differentiated. SMX had a wide range of identified TPs, there were 11 observed in total referred to as SMX_M_215–301. Five of the identified metabolites have also been previously observed and documented, these were SMX_M: 287, 269, 239, 173 and 215, whilst both SMX_M_239 and SMX_259 have not been previously identified.

The transformation products that were identified to have differing rates of formation and degradation within the pH treatments for non-sterile pig slurries are presented in Fig. 2, please refer to SI Figs. 3–10, for other TP profiles. The spectrum of TPs for FLO reveals pig slurry pH to affect the timing of TP formation as well as the intensity detected, this resulted in differences in TP degradation rates and routes (Figs. 2–3).

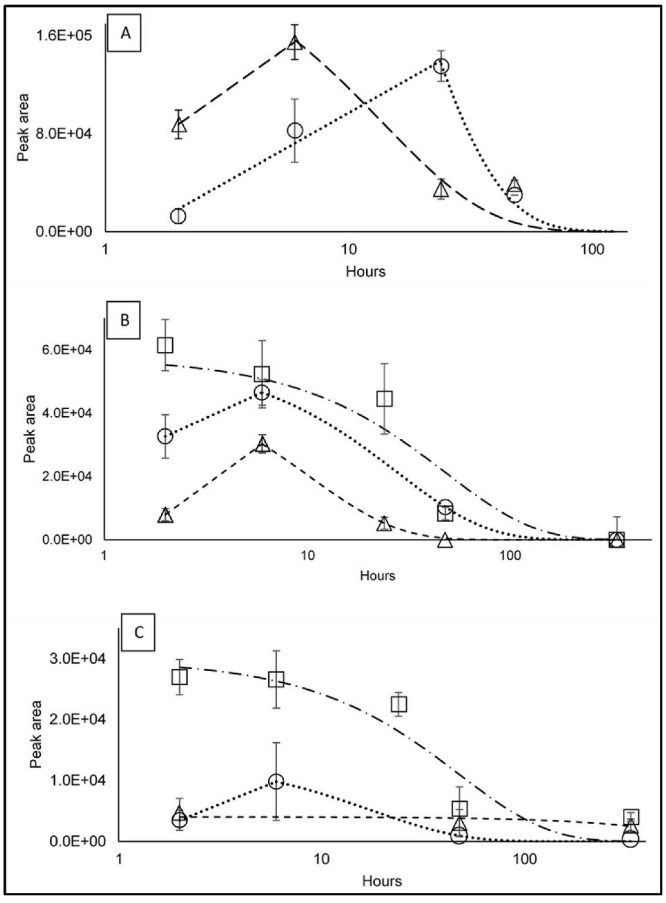


Fig. 2. *Kinetics of identified TPs in non-sterile slurries as* per *slurry* pH *treatments over time, metabolite intensity peak area is plotted on the Y-axis and time on the X-axis.*Key: pH 8.5 pH 7 pH 5.5 A - Monochloroflorfenicol, B – TYL_B, C - FLO_M_338.

Generally, under pHs 7–8.5 FLO degraded into MCF rapidly whereas under pH 5.5 FLO was observed to form FLO_M_338 and very little MCF. As a result of faster FLO degradation under alkaline conditions (Fig. 1), rapid formation of MCF was observed (Fig. 2). Interestingly the *k* rate constants were similar (pH 8.5 *k* 0.0753 and pH 7 *k* 0.07395), which produced DT_{50} values similar to one another (SI Table.5). Rapid and greater intensity of FLO_M_338 formation was observed within acidic slurries, this resulted in a DT_{50} that was over five times that of the neutral treatment (6.4–33 h) (Fig. 2). TYL transformation into TYL_B was found to be rapid at a pH of 5.5 (Fig. 2), the calculated DT_{50} was found to be two times higher than that of neutral (13.4–31.4 h). SMX degradation produced a wide spectrum of both known and unknown TPs (SI Table.4), the formation of these was observed not to be pH dependent (SI Figs. 5–8).

4. Discussion

4.1. Antibiotic degradation as a function of pH

CFT, FLO, TYL and SMX were all observed to be impersistent within pig slurries at a pH of 7 and the DT_{50} values were in-line with previous reports of degradation rates within pig slurries/manures and anaerobic digestion (Gilbertson et al., 1990; Hollis, 1991; Mohring et al., 2009; Chatterjee et al., 2013; Berendsen et al., 2018; Junker et al., 2020). Acid or alkaline treatments were observed to increase/decrease degradation rate constants. Although OTC is known to be environmentally persistent (Blackwell et al., 2005; Junker et al., 2020), Wang et al. (2015) reported a DT_{50} value of 9.05–9.65 d, which we speculate to be related to the acidic pH (pH 5.6) and low moisture content of the pig manure used. The persistence of OTC within pig slurries is likely a result of adsorption mechanisms (cation exchange and electrostatic forces) which render it unavailable to hydrolytic and microbial degradation processes (MacKay and Canterbury, 2005; Blackwell et al., 2005). A similar pH effect for TYL has been observed within dairy lagoon sediment, comparable to those findings presented here, with alkalinity favoring degradation and acidic inhibiting degradation (Ali et al., 2013).

4.2. Biotic and abitoic degradation mechanisms

4.2.1. Microbial mineralization

It is evident from the degradation assessments in sterile and non-sterile slurry that biotic degradation is the primary removal mechanism for FLO, TYL and CFT in pig slurries. The differences in degradation rates for these antibiotics at pH 5.5 and 8.5 are speculated to be a result of reduced/increased microbial activity and mineralization. Sommer et al. (2015) reported acidifications of pig slurries reduced methanogen populations by 6–20%, whilst Lin et al. (2015) reported weak alkaline conditions promote anaerobes such as *Porphyromonadaceae* and *Lachnospiraceae*. The sterile-OTC assessment was inconclusive; we speculate that the faster rates of depletion under sterile conditions was attributed to the degradation of formaldehyde into formic acid, likely further acidifying the surface of the organic matter and further increasing adsorption mechanisms (Suresh et al., 2019).

4.2.2. Adsorption and hydrolysis

It is well known that the pH of soils and sediments influences the rate and mechanisms of adsorption for veterinary medicines, for example publications have detailed an increase in the adsorption coefficients at lower pHs for tetracycline, spectinomycin and florfenicol (Hu et al., 2008; Conde-Cid et al., 2019; Wang et al., 2014). Whilst an adsorption study alone was not conducted, the differences between the abiotic - biotic degradation assessments (non-sterile, sterile and hydrolysis) indicate that adsorption processes could have contributed to the differing rates of degradation that was observed between the pH treatments. These assessments revealed that differences within the degradation rates between pH treatments could be related to adsorption

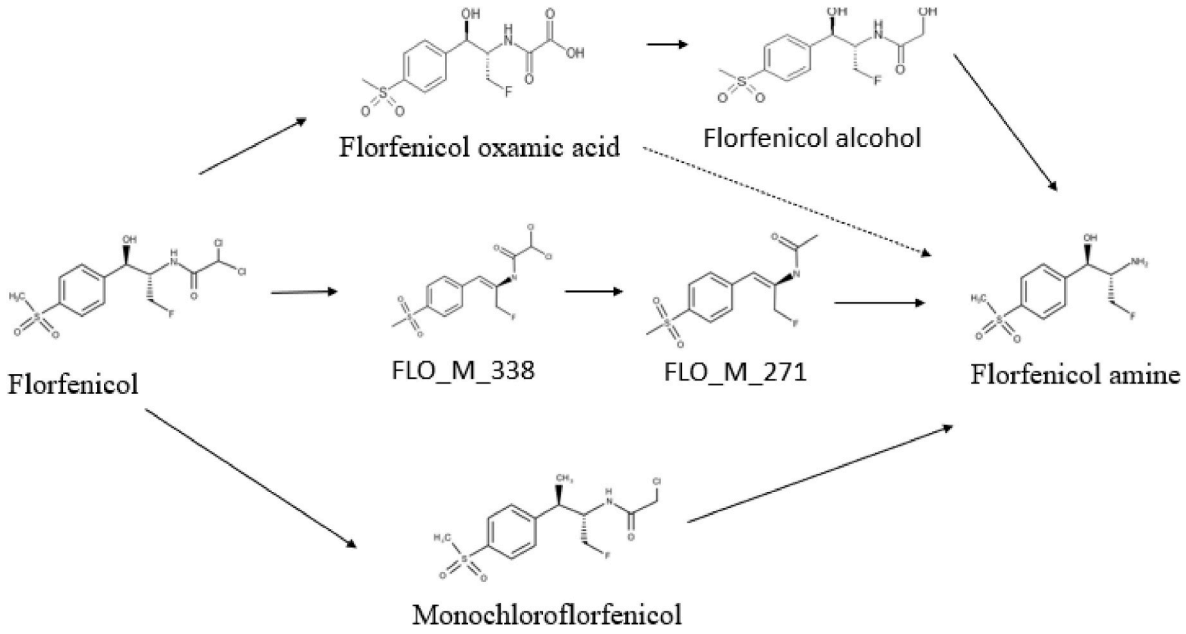


Fig. 3. Purposed degradation pathway of florfenicol in non-sterile pig slurries.

processes for SMX and to a lesser extent TYL (Fig. 1). At lower pHs SMX is an uncharged species (pKa_1 1.4 and pKa_2 5.7), increasing its affinity to form hydrophobic interactions with organic matter/carbon as well as cationic complexes with lipids, carboxylic and hydroxyl groups (Boguta and Sokołowska, 2020; Hu et al., 2019; Jia et al., 2017; Srinivasan et al., 2013). Therefore, we speculate that the increased SMX dissipation at a pH 5.5 and stunted dissipation at pH 8.5 was found to be related to sorption and to a lesser extent hydrolysis. This phenomenon has previously been identified for SMX within acidic composted manures and acidic manured soils. For example, the adsorption-desorption coefficient (K_d) at pH 4 was 2383.3 L/kg whilst at pH 5.5 this was 24.9 L/kg (Hu et al., 2019; Thiele-Bruhn and Aust, 2004). Moreover, the accelerated hydrolysis rate at lower pHs is a result of increased protonation of SMX when it is predominantly in its cationic form (Manzo and Martinez de Bertorello, 1978; Białk-Bielińska et al., 2012). ter Laak et al. (2006) demonstrated that at pH 8.5 the adsorption coefficient of TYL increases, therefore it is highly likely that the observed differences within the degradation rate under the non-sterile degradation assessment are related to the promotion of adsorption mechanisms.

4.2.3. *Transformation products within slurry treatments*

It is critical to consider TPs when assessing the degradability of compounds within environmental matrices as in some cases these products can maintain a similar mode of action, as well as having similar or greater ecotoxicological effects, and persistence within the environment (Sinclair and Boxall, 2003; La Farre et al., 2008; Koba et al., 2017). For example, florfenicol alcohol (FOH) has been reported to have similar toxicological effects towards the green algae *Pseudokirchneriella Subcapitata* as FLO; the minimum inhibitory concentrations for these were found to be $>$ 0.98 mg/L (FDA, 2013; Hoberg, 1991). Increasing attention is being given towards TPs within recent publications,however, due to cost and availability of analytical standards and LC-HRMS, our understanding of the behavior of TPs in the environment is still in its infancy.

Differences within degradation rates for CFT could be related to the formation of desfuroylceftiofur and its analogue deacetyl-cefotaxime. These TPs form through the cleavage of thioester bonds and it has been identified that this occurs more rapidly under alkaline conditions (Koshy and Cazers, 1997; Sunkara et al., 1999). The rapid degradation of FLO under alkaline/neutral conditions was found to be a result of biotic transformation into MCF. MCF is unstable and degrades into the final degradant florfenicol amine (DT_{50} 35 h), although no clear relationship was observed between the pH treatments, MCF and florfenicol amine (FA) (Fig. 3). Higher intensity detections were observed for both FOA and FLO_M_338 within the acidic slurries, however it remains unclear whether increased formation was observed within these conditions or inhibited degradation provided better detections of these TPs. FLO_M_271 is potentially a degradant of FLO_M_338 and both have previously identified within agricultural soils, but this is the first time they have been identified within pig slurries (Qiu et al., 2021).

Nine metabolites were identified during the degradation of SMX, five of which have previously been identified and detected within sewage sludge and hospital wastes (Martín de Vidales et al., 2012; Srinivasan et al., 2013). SMX's TP profile is complex but indicates that all pH treatments had the same TPs present (SI Figs. 5–8). Although higher rates of formation and faster degradation rates were observed at different pHs, there was little compelling evidence to suggest that the acidic slurry treatment increased the rate or altered the route of SMX transformation. TYL_B has been previously detected within animal tissues, slurries and manured soils and is known to form under acidic conditions (Loke et al., 2000; Cherlet et al., 2002). The transformation product profile of TYL did not reveal a suitable explanation to increased removal within the alkaline treatment although we suspect this to be related to unidentified TPs that were not detected during the analyses.

4.2.4. *Scientific importance and implications towards the management of manures and the veterinary medicine risk assessment*

The processing of animal slurries is often overlooked within veterinary medicine risk assessment even though farmers frequently adjust the properties of slurries (acidification and moisture) in order to improve/retain nutrient content, reduce greenhouse gas emissions and improve compliance with the Nitrate Directive (91/676/EEC) (Kai et al., 2008; Clemens et al., 2002; Sommer et al., 2017; Wang et al., 2014). Slurry acidification is becoming increasingly popular in Holland and Denmark due to the reduced requirement to plough or inject slurries from Dutch authorities; in 2012 10% of slurries were acidified (Hjorth et al., 2013; Fangueiro et al., 2015a).

Until now the influence of acidifying slurries on antibiotic degradation was unknown but the results presented here demonstrate this could lead to an exceedance of permissible limits of antibiotics in the

environment deemed as acceptable under current risk assessment paradigms. For example, if the measured fate data (DT_{50}) were used to derive $PEC_{soil\ refined}$, the slurry acidification scenario would result in increased soil concentrations of FLO and TYL (EMA, 2011). The $PEC_{soil\ refined}$ for TYL at a pH of 7 is 45 times lower than that when using the pH 5.5. Similarly, FLO $PEC_{soil\ refined}$ is 1.23E-32 μg/kg at a pH 7, but at pH 5.5 this was calculated to be 138.21 μg/kg. If degradation was assessed at an acidic pH, the PEC calculation is in an excess of the specified 100 μg/kg threshold (EMA, 2016). Elevated concentrations also present wider ecosystem risks that would not have been considered if degradation was assessed at neutral pH. For example, increased concentrations of TYL within arable soils would decrease post-emergence survival for all of the standardized plants assessed via Simon et al. (2015) (EC_{50} 15–400 mg/kg). Likewise, the elevated FLO soil concentrations have previously been identified to cause 10% decrease in the biomass for *A. cepa, B.napus* and, *S.alba* (EC_{50} 60 to >70 μg/kg) (Richter et al., 2016). Comparatively, the acidification process would be an ideal mitigation measure for removing SMX from pig slurries and soils.

In order to rectify variability within manure degradation trials, we suggest that multiple slurries of the same type with a range of properties should be used, which will provide a more thorough assessment of veterinary medicine degradability. It is probable that similar effects of pH seen in the correct investigation maybe observed in cattle manure and poultry litter, but the impact of pH on degradation rate and route of antibiotics has not been determined yet. To ensure the subsequent environmental risk assessment is comprehensive and precautionary then it is important that manure degradation studies replicate realistic on-farm practices and variability. Therefore, we suggest a range of pig slurries are considered which as a minimum demonstrate a range of realistic pH values.

5. Conclusion

The crucial findings of this study demonstrate the influence of pig slurry pH on the dissipation of veterinary antibiotics and could contribute to a more robust risk assessment. A compound specific effect was found for antibiotic degradation and the effect of slurry pH, for example, FLO, TYL and CFT degradation was inhibited under acidic conditions and enhanced under alkaline over that of neutral. SMX degradation was observed to increase under acidic conditions and be inhibited under alkaline over that of neutral. Biotic processes drove differences between pH treatments for FLO, TYL and CFT, whilst abiotic processes contributed to differences for SMX and to a lesser extent TYL. Different TPs were identified associated with particular pH conditions, revealing differences within biotic processes. The findings presented here demonstrate that current regulatory and manure management practices could result in greater environmental exposure than anticipated.

SUPPORTING INFORMATION: Additional experimental methods for characterizing pig slurries, information regarding quality control, experimental design, experimental dosage concentrations, extraction validation, additional mass spectrometer details, identified transformation products (mass and structures), collated DT_{50} values for transformation products, hydrolysis kinetic figures and transformation product kinetics.

Author contributions

John Nightingale: Laboratory methods, conceptualization, analytical chemistry, statistical analysis, and writing/editing, Laura Carter: Funding acquisition, expert experimental guidance, methodology, conceptualization, reviewing data, statistics, and writing/editing, Paul Kay: Funding acquisition, expert experimental guidance, methodology, reviewing data and writing/editing, Chris Sinclair: Funding acquisition, expert guidance on manure degradation and the risk assessment, conceptualization, experimental methods, experimental design, reviewing data and writing/editing, Philip Rooney: Expert guidance on manure degradation, funding acquisition, degradation kinetics, and reviewing data, Michael Dickinson: Expert guidance on LC-HRMS, training to use LC-HRMS, reviewing transformation products and writing/editing, Jonathan Tarbin: Database for possible phase I and phase II transformation products.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2021.133191.

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