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Environmental fate and microbial effects of monensin, lincomycin, and sulfamethazine residues in soil[☆]

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ABSTRACT

The impact of commonly-used livestock antibiotics on soil nitrogen transformations under varying redox conditions is largely unknown. Soil column incubations were conducted using three livestock antibiotics (monensin, lincomycin and sulfamethazine) to better understand the fate of the antibiotics, their effect on nitrogen transformation, and their impact on soil microbial communities under aerobic, anoxic, and denitrifying conditions. While monensin was not recovered in the effluent, lincomycin and sulfamethazine concentrations decreased slightly during transport through the columns. Sorption, and to a limited extent degradation, are likely to be the primary processes leading to antibiotic attenuation during leaching. Antibiotics also affected microbial respiration and clearly impacted nitrogen transformation. The occurrence of the three antibiotics as a mixture, as well as the occurrence of lincomycin alone affected, by inhibiting any nitrite reduction, the denitrification process. Discontinuing antibiotics additions restored microbial denitrification. Metagenomic analysis indicated that Proteobacteria, Bacteroidetes, Actinobacteria, and Chloroflexi were the predominant phyla observed throughout the study. Results suggested that episodic occurrence of antibiotics led to a temporal change in microbial community composition in the upper portion of the columns while only transient changes occurred in the lower portion. Thus, the occurrence of high concentrations of veterinary antibiotic residues could impact nitrogen cycling in soils receiving wastewater runoff or manure applications with potential longer-term microbial community changes possible at higher antibiotic concentrations.

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1. Introduction

Although nutrient runoff and pathogen risks associated with livestock manures have been long recognized, the environmental risks associated with incompletely metabolized pharmaceutical compounds used to treat livestock and excreted in manure were not recognized as a potential environmental issue until recently. Antibiotics have been detected in multiple agricultural sources (i.e., livestock wastewater, manure and crops fertilized with manure) (Aust et al., 2008; Bartelt-Hunt et al., 2011; Brown et al., 2006;

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Burkholder et al., 2007; Sim et al., 2011; Watanabe et al., 2008; Wei et al., 2011; Wei et al., 2016). Confined animal feeding operation (CAFO) may locally affect surface and groundwater quality. For example, sulfamethazine (0.076–0.22 $\mu\text{g L}^{-1}$) and sulfadimethoxine (0.046–0.068 $\mu\text{g L}^{-1}$) were detected in all six groundwater samples collected near a CAFO in Weiser, Idaho (Batt et al., 2006). In Nebraska, sulfamethazine, monensin, lincomycin, sulfathiazole, and sulfamerazine have been detected in samples from cattle and swine facilities as well as in agriculturally intensive watersheds (Bartelt-Hunt et al., 2011; Jaimes-Correa et al., 2015; Snow et al., 2011; Zhang et al., 2013). The activities of soil and groundwater bacteria can be impacted by residues of antibiotics (DeVries and Zhang, 2016). Mobilization of antibiotic residues through the vadose zone and into the aquifer will be subject to the different redox conditions occurring in the environment and also impacting the persistence of both the antibiotic and nitrate (Chee-Sanford

et al., 2009).

Land applied fertilizers and manure contribute to nitrate loading in groundwater beneath agricultural areas (Exner et al., 2014) and also contribute substantially to nitrogen loading in surface water (Spalding and Exner, 1993). High concentrations of groundwater nitrate have been observed, particularly in the shallow aquifers of Nebraska (Exner et al., 2014; Exner and Spalding, 1985; Spalding and Exner, 1993). Sources of nitrate in ground water may be related to manure and chemical fertilizer application beyond crop needs (Buochard et al., 1992; Hepperly et al., 2009; Spalding and Exner, 1993). In addition, reduced forms of nitrogen present in manure (i.e., urea, organic nitrogen, and ammonium) can be mineralized and consequently oxidized to nitrate.

Recent studies have evaluated the impact of different classes of antibiotics (i.e. aminoglycoside, fluoroquinolone, sulfonamide, tetracycline, etc.) on denitrification using soil (Conkle and White, 2012; DeVries et al., 2015), sediment (Costanzo et al., 2005; Hou et al., 2014) and groundwater (Ahmad et al., 2014; Underwood et al., 2011) as media. Results from these studies showed contrasting behavior since antibiotics can have no effect (Conkle and White, 2012; Costanzo et al., 2005; Radl et al., 2015), enhance inhibition (DeVries et al., 2015), or enhance stimulation (Ding and He, 2010; Ilhan et al., 2011; Yan et al., 2013; Yin et al., 2017). Experimental conditions between these studies were different, since antibiotics were added individually (Roose-Amsaleg et al., 2013; Rosendahl et al., 2012) or as a mixture (Conkle and White, 2012; Costanzo et al., 2005; Underwood et al., 2011), their exposure period ranged between hours (Costanzo et al., 2005; Hou et al., 2014) and weeks (Kotzerke et al., 2008; Roose-Amsaleg et al., 2013; Rosendahl et al., 2012), while their concentrations were usually greater than 1 mg L^{-1} (Banerjee and D'Angelo, 2013; Kotzerke et al., 2008; Laverman et al., 2015; Radl et al., 2015). For example, sulfadiazine can stimulate or inhibit denitrification if present at low or high concentrations, respectively (DeVries et al., 2015). Short application (<4 days) of narasin can stimulate denitrification, while long application of the same ionophore antibiotic can inhibit denitrification (DeVries et al., 2015).

The objectives of this study were to determine: i) the environmental fate of three commonly occurring manure-borne veterinary antibiotics (monensin, lincomycin and sulfamethazine) in three common soil redox environments in the presence and absence of nitrate; ii) the effect to microbial respiration and denitrification; and iii) the overall impact to soil microbial communities using next generation sequencing methods. In addition, a comparison of each antibiotic and a mixture of all three to microbial denitrification at environmental concentrations were also investigated.

2. Materials and methods

2.1. Chemicals

Monensin (95% pure), lincomycin (95% pure), potassium nitrate (KNO_3), potassium bromide (KBr), Optima-grade methanol (ThermoFisher, St. Louis, MO, USA), sulfachloropyridazine (Sigma-Aldrich, St. Louis, MO, USA), and sulfamethazine (98% pure, MP Biomedicals, LLC, Solon, OH, USA) were used. Reagent grade deionized (DI) water (Thermo-Scientific, Waltham, MA, USA) with a resistivity of $0.182 \text{ M}\Omega \text{ m}$ (at 25°C) was used. The chemical properties of the three antibiotics are listed in Table S1.

2.2. Soil and water samples

Disturbed soil samples were collected at the 6000-animal capacity, open-air cattle feedlot at the USDA, U.S. Meat Animal Research Center located in Clay Center, south-central Nebraska.

Detailed description of the facilities can be found elsewhere (Parker et al., 1999; Zhang et al., 2013). The soil is classified as Crete silt loam (fine, smectitic, mesic Pachic Argiustolls containing 24% clay, 7% sand and 69% silt with 3% organic matter) (USDA, 2012). Surface soil samples were collected using a hand shovel at 5–10 cm beneath the surface, from an open-air feedlot at the drainage ditch immediately down-slope from the feedlot pen. Soil samples were air-dried, ground with a pestle to break the aggregates, sieved (<2 mm), and stored at 4°C prior to being used. Feed water for the soil columns was collected from a runoff retention basin at the same feedlot. Surface water samples were collected approximately 1 m away from the bank of the basin and stored at 4°C prior to being used.

2.3. Experimental set-up

Vertical flow-through columns (5 cm inner diameter; 16 cm height) were used during the study. The columns had opaque walls to reduce the photo-degradation of the selected antibiotics. At the bottom of each column, a stainless steel wire mesh (openings: $40 \mu\text{m}$; TWP, Berkeley, CA, USA) was placed to support the added soil and to prevent any potential leaching of the fine particulates. The wire cloth as well as all the different fittings and tubing used during the study were sterilized by autoclaving (STERIS, Mentor, OH, USA) for 30 min at 121°C and 143.4 kPa. Each column had two stainless steel sampling ports ($5 \mu\text{m}$ openings, Mott Corporation, Farmington, CT, USA) horizontally inserted through the unit wall and located 1.5 and 8 cm from the top.

Soil was added in increments of 5 mm to each column and compacted using a vortex mixer (VWR, Thorofare, NJ, USA) until the original bulk density was achieved. This process prevented the occurrence of macropores in the columns. Columns were slowly saturated from beneath to minimize air entrapment. A four channel head peristaltic pump (Cole-Parmer; Vernon Hills, IL, USA) was used to simultaneously introduce the different feed solutions to the different columns at a steady-state flow of 14 cm d^{-1} . Each column was acclimated for 14 d using a mixture of filtered feedlot runoff and DI water (10:90). The feed water was autoclaved (STERIS, Mentor, OH, USA) at 121°C and 143.4 kPa for 30 min before being used. After acclimation, a solution containing the three antibiotics (either singly or in combination – $20 \mu\text{g L}^{-1}$) and bromide tracer (100 mg L^{-1}) in feed water was applied for 7 d (referred to as “spike exposure”). After the injection of antibiotics was suspended, each column was flushed with feed water alone for approximately 30 d (referred to as flush-out).

The effect of oxygen— aerobic versus anoxic versus denitrifying—on antibiotic transport and transformation was investigated by performing the different simulations at a level of oxygen $>8 \text{ mg L}^{-1}$ (aerobic), or $<2 \text{ mg L}^{-1}$ (anoxic), or $<2 \text{ mg L}^{-1}$ plus nitrate (denitrifying). Aerobic conditions were maintained by keeping the reservoir open to the atmosphere and bubbling air, while reducing/denitrifying conditions were obtained by keeping the reservoirs closed and by continuously purging the reservoir head-space and sparging the feed water with nitrogen (99% purity – Matheson Tri-Gas, Lincoln, NE, USA). Denitrifying conditions were obtained by continuous addition of 10 mM KNO_3 , and fresh feed water solutions were prepared three times per week in order to prevent any nitrate/nitrite transformation in the feed water and maintained at 4°C . An additional column, packed with sterile packing material and fed with sterile water, was used as control to evaluate the chemical removal versus biodegradation of the three antibiotics. The highest impact of biodegradation was expected to be under aerobic conditions, therefore the control column was run only under aerobic conditions. Feed water, column, sampling ports and fittings were sterilized by autoclaving at 121°C and 143.4 kPa.

for 30 min. Soil used as packing material, similar to the material used throughout the study, was autoclaved at 121 °C and 143.4 kPa for 30 min for three consecutive days before being used. Table S2 summarizes the environmental conditions investigated during the study.

To better understand the effects of the antibiotics on microbial community composition, a subsequent series of incubations simulating aerobic anoxic, and denitrifying environmental conditions were conducted where columns were sacrificed at specific points during the incubation. Columns were run either i) for 14 days (acclimation period), ii) for 21 days (14-day acclimation period followed by a 7 day antibiotics injection), or iii) for 54 days (14-day acclimation period, 7-day injection of antibiotics, and 33-day flush-out) and then sacrificed and frozen for subsequent DNA extraction and analysis. Table S3 summarize the different experimental conditions used throughout the study, their duration, the presence or absence of antibiotics and the individual objectives.

Prior to DNA extraction, soil samples were divided into the upper (1.5 cm), middle (8 cm), and lower (15 cm) portion of each column and cross-sectioned into 80 quadrants of 0.5×0.5 cm. Eight quadrants were randomly sampled with a sterile spatula and mixed to provide a representative sample. An unexposed subsample of soil and feed water sample were also analyzed. Total DNA was extracted from the soil samples using the MoBio Power Soil® DNA Isolation Kit (MoBio, Laboratories Inc, Carlsbad, CA, USA) following manufacturer's instructions. The total microbial DNA extractions were subjected to 16S ribosomal RNA sequencing, using the Ion Torrent PGM sequencing platform. Sequencing was performed by Molecular Research Laboratory (Shallow Water, TX, USA) using universal eubacterial primers 515F and 806R (Dowd et al., 2008). Samples were purified using Agencourt Ampure beads (Agencourt Bioscience, MA, USA), barcodes were assigned, samples were applied to capture beads, and sequencing was performed according to manufacturer's instruction. Initial quality control measures included demultiplexing and denoising of flowgrams, and removal of adapters, barcodes, primers, short reads, and homopolymers. Next, sequences for individual samples were aligned, and chimeric sequences were flagged and removed from the dataset, along with amplicons classified as mitochondrial, chloroplast, archaeal, eukaryotic or unknown in origin. A secondary file was generated with consensus sequences, and this consensus sequence was used as input for a BLASTn search (Altschul et al., 1990), against a database assembled from RDP (Maidak et al., 2001) and GenBank. E-values were used to determine the best taxonomic assignments at the >98% similarity level. Sequences were grouped by taxon, descriptive statistics on percent of each taxon present in each sample were calculated in Excel, as was the Shannon Diversity index for each sample. A list of genera containing denitrifying members (at least one confirmed denitrifier in the genus) was assembled by Zumft (1997), and a separate analysis using only these genera was performed for the samples under denitrifying conditions.

2.4. Sampling and chemical analysis

Influent, effluent, and port samples were collected and analyzed for major anions (chloride, sulfate, orthophosphate, nitrate, nitrite, and bromide), and selected antibiotics (lincomycin, monensin, and sulfamethazine). Samples from the two sampling ports, horizontally inserted through the external surface of each column, were collected by allowing the water to slowly flow through a valve connected to each port.

Analyses of selected antibiotics were performed using an Agilent 1260 binary high pressure liquid chromatograph (HPLC) coupled to an Agilent 6410 Triple Quadrupole mass spectrometer

(Agilent Technologies, Santa Clara, CA, USA). Antibiotics were separated using a HyPURITY C18 HPLC column (250 mm $\times$ 2.1 mm ID, 5 μ m particle size, Thermo-Scientific, Waltham, MA, USA) at a temperature of 50 °C. The HPLC gradient at flow rate of 0.20 mL min^{-1} began with 0:100 (methanol-0.01% formic acid:water-1 mM ammonium citrate) held for one minute then a linear gradient to reach 80:20 over a period of two minutes and a 95:5 over a period of nine minutes, hold for 9 min before resetting (0:100) and equilibrating for five minutes. Ionization of analytes was performed with positive mode. A pseudo-molecular ion $[M+H]^+$ (or $[M+Na]^+$ adduct) was selected as the parent ion for fragmentation, and corresponding fragment ion(s) was selected for identification and quantitation. Fragmentor and collision energies used for each standard and analytes are given in Table S4, while statistics associated with the used methodology are given in Table S5. Samples were filtered using a Glass Microfiber syringe filter (0.45 μ m, Whatman, GE Healthcare Life Sciences, Pittsburgh, PA, USA) and sulfachloropyridazine, surrogate used during the study, was added. Surrogate had a recovery of $81.64 \pm 4.17\%$. Recovery of the selected antibiotics was estimated by summing the mass removed after each column (effluent) or after the two sampling ports and dividing by the total mass injected. Major anions were measured with an ion chromatograph (ICS-90) (Dionex, Bannockburn, IL, USA) with a detection limit of 0.1 mg L^{-1} .

Redox potential (Eh) in the effluent was continuously monitored using HYPNOS III logger (MVH Consult, Leiden, The Netherlands). The device incorporates a control unit, a multiplexer, storage memory and redox probes (Vorenhout et al., 2011). The redox probes consisted of a fiberglass shell, having a diameter of 8 mm, equipped with platinum wire tips that were partially covered in the fiberglass (PaleoTerra, Amsterdam, The Netherlands), which were placed in flow-through cells.

3. Results and discussion

3.1. Column redox conditions and general chemistry

Measured effluent in one column was maintained under aerobic conditions ($\text{DO} > 8 \text{ mg L}^{-1}$, Eh between 480 and 600 mV), while under anoxic condition, Eh decreased to suboxic conditions ($100 < \text{Eh} < 400 \text{ mV}$) during the first day and prior to reach anoxic conditions ($\text{Eh} < 100 \text{ mV}$) within two days. Under denitrifying conditions, Eh showed a similar but slower trend compared to the trend observed under anoxic conditions. After ten days, the column receiving 10 mM KNO_3 maintained a denitrifying Eh consistently $< 100 \text{ mV}$ (Fig. S1). Under aerobic, anoxic, and denitrifying conditions, effluent pH ranged between 7 and 8, 6.9 and 9, 7.2 and 9, respectively (Fig. S2). In the presence of denitrifying conditions, hydroxide ion is generated and consequently higher pH values are expected. Electrical conductivity (EC) rapidly increased during the first two days, decreased during the next three days and plateaued after that (Fig. S3). The rapid EC increase was related to the rapid leaching of anions (i.e., sulfate and orthophosphate) from the soil after receiving a dilute and sterile mix of 10:90 feedlot runoff and DI water.

3.2. Fate of antibiotics

Fractional (C_i/C_0) breakthrough curves (BTCs) of the three antibiotics are shown in Fig. 1 and could be related to the chemical properties of the antibiotics. Peak breakthrough occurred earlier for the bromide (Fig. S4) than for the antibiotics (Fig. 1). The appearance and disappearance of the three antibiotics in the effluent and from the two sampling ports was slow, the shape of the BTCs was asymmetric with long tailing (Fig. 1), characteristic of desorption

Fig. 1. Breakthrough curves (BTCs) of three antibiotics: (a) lincomycin, (b) monensin, and (c) sulfamethazine under aerobic ($\text{DO} > 8 \text{ mg L}^{-1}$), anoxic ($\text{DO} < 2 \text{ mg L}^{-1}$), and denitrifying ($\text{DO} < 2 \text{ mg L}^{-1}$ and KNO_3) conditions. Antibiotics ($20 \mu\text{g L}^{-1}$) and bromide (100 mg L^{-1}) were added between day 14 and day 21. BTCs under aerobic, anoxic, and denitrifying are red, blue, and green, respectively. Rhombus, triangle, and circle represent BTCs from the first sampling port (1.5 cm), the second sampling port (8 cm) and effluent, respectively. Open symbols and dashed line represent the experimental control under aerobic conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and dispersion. Among the three antibiotics, monensin was not detected in any samples from the column effluent and only detected in pore water samples collected in the upper portion of the columns regardless of the environmental (aerobic vs. anoxic vs. denitrifying) conditions. Lincomycin and sulfamethazine were detected in the effluent throughout the study, but effluent concentrations decreased during transport through the soil columns (Fig. 1).

Mass recovery (the ratio of the mass recovered after the two sampling ports and in the effluent compared to the mass injected) decreased with depth with lincomycin and monensin showed the highest and the lowest mass recovery, respectively (Table S6). Under aerobic and anoxic conditions, lincomycin was completely recovered (100% of injected concentration) in the upper half of each column (up to 8 cm), and decreased, in the lower half of each column, to 68% and 53% under anoxic and aerobic conditions, respectively. Under denitrifying conditions, mass recovery of lincomycin decreased from 100% (upper 1.5 cm) to 68% (upper 8 cm) to 28% (lower half of the column) (Table S6). Recovery of sulfamethazine and lincomycin was lower under denitrifying conditions than under aerobic and anoxic conditions. This behavior may be linked to the change in microbial communities observed under denitrifying conditions, resulting in an increasing removal of the two antibiotics and on a significant impact on the nitrate/nitrite cycle. Bertelkamp et al. (2015) investigating the fate of twenty organic micropollutants under anoxic conditions, observed long half-life, and expected higher removal for lincomycin, and consequently lower mass recovery, under more reducing conditions and in the presence of a longer residence time. Sulfamethazine showed a similar behavior, but slightly lower mass recovery (~60% under aerobic and anoxic conditions) compared to lincomycin. Park and Huwe (2016) investigating the effect of solution pH and soil

structure on transport of sulfonamide compounds using soil columns, achieved 67–85% recovery of sulfamethazine under aerobic conditions. Limited mass recovery of monensin was observed throughout the study. In the upper 1.5 cm, mass recovery ranged between 22% under anoxic conditions to ~8% under denitrifying conditions (Table S6). Concentrations of the three antibiotics showed a similar behavior compared with the results obtained under aerobic sterile conditions (Fig. 1), suggested that biodegradation played a minor role.

Monensin's hydrophobic nature ($\log K_{ow}$ of 5.43) contributed to its observed low mobility (Table S1); it was only observed within the top 1.5 cm of the column with low to no recovery in the effluent. Limited movement of monensin was observed in a field study conducted by Carlson and Mabury (2006). Strong sorption of monensin to soils and sediments is expected due to its hydrophobicity. Sassman and Lee (2007) observed high sorption of monensin on different agricultural soils. These results are in accordance to previous studies on monensin disappearance in soils (Carlson and Mabury, 2006; Sassman and Lee, 2007; Watanabe et al., 2008), suggesting that monensin would degrade primarily in agricultural soils before moving to groundwater. In addition, at high values of pH, monensin is neutralized by chelating with cations and forms complexes which are more likely to increase the $\log K_{ow}$ of the ionized monensin, and therefore its hydrophobicity and its sorption capacity (ElSayed and Prasher, 2014). On the other hand, lincomycin and sulfamethazine are classified as hydrophilic ($\log K_{ow}$ of 0.29 and 0.76, respectively) and thus were expected to migrate through the soil columns. In addition to hydrophobicity, pH likely also affects mobility. The pK_a values of lincomycin, monensin, and sulfamethazine are 7.6, 4.2, and 7.65, respectively (Table S1). Effluents from the soil columns showed pH values ranging between 7 and 9, greater than pK_a of selected antibiotics, suggesting enhanced

movement of lincomycin and sulfamethazine. The mobility of lincomycin and sulfamethazine is enhanced by pH values above 7.5. In particular, the increased mobility of sulfamethazine at high pH is due to increased presence of anionic sulfamethazine (speciation of approximately 65%) and consequently sorption of sulfamethazine to negatively charged mineral and organic matter surfaces is expected to decrease (Boxall et al., 2002; Chu et al., 2013; Gao and Pedersen, 2005; Kahle and Stamm, 2007; Park and Huwe, 2016; Thiele-Bruhn and Aust, 2004). Relatively low values of the adsorption coefficient were observed during batch investigations supporting the mobility of sulfamethazine in soil (Chu et al., 2013; Doretto et al., 2014). Similarly, to sulfamethazine, enhanced movement and consequently reduced sorption of lincomycin occurs while pH solution increased. Wang et al. (2009) observed a reduced sorption of lincomycin while pH solution increased.

3.3. Effect of antibiotics to N transformation

Feed water was intentionally spiked with nitrate in the denitrifying treatment. Thus, very little nitrate or nitrite was observed in the effluent of aerobic and anoxic columns (Fig. 2). No nitrate/nitrite transformation was observed in the feed water reservoir during the study. During the acclimation period (no antibiotics added to the feed water), nitrate increased from 10 to 75 mg L⁻¹ in the effluent, while nitrite ranged between 5 and 25 mg L⁻¹, during the same period (Fig. 2). During acclimation, stable nitrate concentrations were observed at all sampling intervals and overall decreased by roughly half as nitrate-amended feed water moved through the soil column. Nitrite was not observed in the upper portion of the column, but a small amount was consistently detected in the effluent during acclimation. After the injection of the three antibiotics in the feed water, nitrate concentrations decreased quickly in the first two sampling ports and eventually decreased below the analytical detection limit after 6 days of injection (Fig. 2a). In parallel, nitrite concentrations began to increase and reached a maximum of 60 and 30 mg L⁻¹ at the end of the spike at the first and second sampling port, respectively (Fig. 2b). After discontinuing influent antibiotic dosing, nitrate concentrations increased and nitrite concentrations decreased to acclimation period levels (Fig. 2). A test to compare individual versus mixed antibiotic effect in feed water was used to evaluate the effect to evaluate the effect to microbial respiration (Fig. 3). Among the three antibiotics, monensin alone had a negligible effect while individually added sulfamethazine and even more lincomycin affected the nitrate/nitrite transformation (Fig. 3). In particular, sulfamethazine and lincomycin individually added to the feed water, were able to replicate the impact of the antibiotics' mix on the nitrate/nitrite transformation in the lower half of the columns (Fig. 3b and c). The impact of these two individually added antibiotics was less pronounced in the upper portion of the column (Fig. 3a). In fact, while nitrate was completely degraded during the injection of the antibiotics' mix, a 60% reduction of nitrate was achieved in the upper portion when sulfamethazine and lincomycin were individually added.

The nitrate/nitrite transformation observed throughout the study can be linked to the permanent changes in microbial communities occurred in the upper 1.5 cm, while temporary changes occurred throughout the column (Fig. 4, S5). In the upper 1.5 cm, Actinobacteria (~4%) and Alphaproteobacteria (~7%) progressively decreased while Sphingobacteriia (+4%) progressively increased (Fig. S5). This behavior – long term changes in top 1.5 cm vs. temporary changes in the lower portion – was likely linked to higher exposure to antibiotics in the upper portion of the column compared to the lower portion. Among the three antibiotics, lincomycin had the most similar effect on the microbial

Fig. 2. Impact of selected antibiotics on (a) nitrate and (b) nitrite under aerobic ($\text{DO} > 8 \text{ mg L}^{-1}$), anoxic ($\text{DO} < 2 \text{ mg L}^{-1}$), and denitrifying ($\text{DO} < 2 \text{ mg L}^{-1}$ and KNO_3) conditions. During the “Acclimation”, the column received an autoclaved mixture of feedlot runoff (10%) and deionized water (90%); during the “Spike”, $20 \mu\text{g L}^{-1}$ mix of lincomycin, monensin, and sulfamethazine were added; during the “Flush Out” the injection of the mix of antibiotics was suspended. Breakthrough curves (BTCs) under aerobic, anoxic, and denitrifying are red, blue, and green, respectively. Rhombus, triangle, and circle represent BTCs from the first sampling port (1.5 cm), the second sampling port (8 cm) and effluent, respectively. Open symbols and dashed line represent the experimental control under aerobic conditions. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

communities throughout the columns compared to the antibiotic mix (Fig. S6).

Results from this study confirmed that sulfamethazine affects nitrate transformation to nitrite, the first step of the denitrification process (Ahmad et al., 2014; Hou et al., 2014; Yin et al., 2017). Ahmad et al. (2014) investigated the inhibitory effect of antibiotics on denitrification using microcosms experiments and observed enhanced removal of nitrate and production of nitrite under anaerobic conditions in the presence of sulfamethazine. At relatively low concentration (0.01 mg L^{-1}), sulfamethazine decreased nitrate reduction rates, while higher concentrations (0.1 – 1 mg L^{-1}) stimulated nitrate reduction to nitrite (Ahmad et al., 2014). Similarly to Yin (Yin et al., 2017), multiple antibiotics, including sulfamethazine, enhanced nitrate conversion to nitrite. Sun (Sun et al., 2017) investigating the role of excessive anthropogenic nitrate input in the anoxic dissipation of soil antibiotics, including two sulfa-compounds/ARGs observed a sharp decline in nitrate during the first five days of incubation and an increase in the production

Fig. 3. Impact of selected antibiotics on nitrate under denitrifying ($\text{DO} < 2 \text{ mg L}^{-1}$ and KNO_3) conditions after (a) the first sampling port (1.5 cm), (b) the second sampling port (8 cm) and (c) effluent, respectively. During the “Acclimation”, the column received an autoclaved mixture of feedlot runoff (10%) and deionized water (90%); during the “Spike”, $20 \mu\text{g L}^{-1}$ mix of lincomycin, monensin, and sulfamethazine were added; during the “Flush Out” the injection of the mix of antibiotics was suspended. “Mix” refers to the spike solution containing a mixture of lincomycin, monensin, and sulfamethazine. “Lincomycin”, “monensin”, and “sulfamethazine” refer to the spike of individual antibiotics.

rate of nitrite during the first 10 days. A recent reported study on the effect of monensin residues to nitrogen transformation in soil

showed no effect of monensin on nitrification rates (Konopka et al., 2015). To our knowledge this was the first study reporting the effect lincomycin on microbial respiration and nitrate-nitrogen reduction in a soil system.

3.4. Antibiotic effect to microbial communities — aerobic and anoxic conditions

Microbial community compositions in the column effluent were sampled throughout the course of the experiment, allowing temporal characterization under three respiratory conditions. There were 1,384,820 sequences that passed quality control, with a mean of 38,467 reads per-library (range 26,026–45,421).

In the untreated control soil used as packing material for the different columns, Proteobacteria (36.1%), Actinobacteria (18.8%), Bacteroidetes (15.7%), and Chloroflexi (12.1%) were the predominant phyla, while none of the other phyla were detected at levels greater than 5% (Fig. 4 and S6). After 14 days of acclimatization with sterilized runoff water, phylum-level distributions remained similar for the aerobic and anoxic columns, with only minor decrease in Proteobacteria (28 vs 33%) and increase in Firmicutes (5 vs 9%) in the surface compared to (bottom – 16 cm) soil samples. Shannon diversity increased over the 14-day treatment with the autoclaved runoff used for acclimatization (Table S7). No phylum-level changes were observed in response to mixed antibiotic spike exposure, in either the aerobic or anoxic columns. Addition of the antibiotic mixture had only a minor impact on Shannon diversity in these two columns, with an increase of 0.02, 0.06, 0.01 and 0.07 in the aerobic top, aerobic bottom, anaerobic top, and anaerobic bottom samples, respectively (Table S7).

The top ten most frequently occurring class level taxonomic assignments covered 68 and 75% of all reads in each sample. Subtle but temporally stable differences were observed in class level distribution between the top and bottom of the aerobic column, with a slightly smaller proportion of bottom samples being assigned to Gammaproteobacteria (Fig. 4, S6). This group of bacteria contains a number of important fecal indicator bacteria and human pathogens. The facultative nature of this group explains the differences observed between the top and bottom of the column. No change was observed in class level Gammaproteobacteria distribution following the seven day three-drug spike, however a small decrease was observed following the 33-day flush (Fig. 4). Overall the seven-day mixed antibiotic exposure changed the distribution of bacteria within the top 10 classes (Fig. 4). Following antibiotic discontinuation and flushing, the communities changed again, with pre-spike and post-flush communities more closely resembling each other compared to those of the post-spike (Fig. 4, S6).

OTU assignments (97%) reveal patterns similar to those seen at the class level, with a clear change in response to the antibiotic mixture, and the pre-spike and post-flush samples more closely resembling each other than the post exposure samples. Across all samples, the top ten OTUs represented less than ten percent of the total reads for each sample (8.3–9.3%). OTUs assigned to Gemmatimonadetes dominated all aerobic and anoxic samples except for the anoxic 21-day post-spike sample, in which the most frequently occurring OTUs belonged to actinobacteria. Classes represented in the top 10 OTUs, but not seen with the top 10 class-level taxonomic assignments were Deinococcus, Epsilonproteobacteria, and Flavobacteria.

3.5. Antibiotic effect to microbial communities — denitrifying conditions

Proteobacteria (28.6–41.9%), Actinobacteria (7.7–17.6%),

Fig. 4. Top ten most frequently occurring class level taxonomic assignments for each respiratory condition (a) over the course of the 54-day experiment and (b) for the individual drugs as well as for their mixture at day 21 under denitrifying conditions. All values displayed are for soil samples taken from the surface soil (top 1.5 cm). Values are expressed as a percentage of the total classes for each sample. For example, examining the day 14 aerobic sample, 16% of all of the bacteria in that sample were classified as Actinobacteria, and a total of 75% of the bacteria in that sample were represented in the top 10 phyla listed. For each heat-map, the highest values are represented in red, and the lowest values in green. A clear response by the microbial community to the antibiotic spike can be seen. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Bacteroidetes (8.4–20.0%), Chloroflexi (11.2–15.5%), and Gemmatimonadetes (7.4–13.2%) were the predominant phyla under denitrifying conditions (Fig. S5). The phylum-level communities in the control soil after 14 days of acclimatization with autoclaved runoff under denitrifying conditions had the same phyla as aerobic and anoxic columns, but had proportionately more sequences assigned to proteobacteria in both the top and bottom of the column compared to the other two respiratory conditions. Under denitrifying conditions, permanent changes in microbial communities occurred in the upper 1.5 cm, while temporary changes occurred throughout the column (Fig. 4, S6).

The class-level taxonomic composition of the denitrified column was identical throughout the incubation to that observed for the aerobic and anoxic columns, however the distribution of sequences was different (Fig. 4). The top ten most frequently occurring class level taxonomic assignments covered between 70 and 81% of all reads in the samples incubated under denitrifying conditions, with Alphaproteobacteria assignments being the most frequent in the top 1.5 cm regardless of sample time (Fig. 4). Betaproteobacteria assignments doubled in response to the spike, indicating the ability of members of this group to flourish in the presence of the 3-drug spike. Betaproteobacteria numbers dropped following the flush. Post-flush, Alphaproteobacteria numbers were lower compared to pre-spike (12 vs 17%), while Sphingobacteria, Gemmatimonadetes, Gamma-, Beta- and Deltaproteobacteria were higher (Fig. S5). Comparing the individual antibiotic spikes to the mixture of three antibiotics, lincomycin had the most similar effect on the microbial communities throughout the columns compared to the antibiotic mix (Fig. S6). Other available studies confirmed that lincomycin similarly impacts the soil microbial communities (Čermák et al., 2008; Grenni et al., 2017).

The denitrifying bacteria are metabolically and taxonomically diverse, and a number of genera have been identified containing denitrifying members (Zumft, 1997). Looking at analysis focused on just these genera, and acknowledging that not every member of a genus is a denitrifier, we observed a shift in response to the three-drug spike. *Cytophaga* was the most frequently assigned genus in the pre-spike samples, compared to *Thiobacillus* and *Flavobacterium* in the post-flush samples (Fig. 5). *Cytophaga* is a common

heterotrophic gram negative gliding soil bacteria in the phylum Bacteroidetes. *Flavobacterium* is another gram-negative soil bacteria in the phylum Bacteroidetes, and it is not surprising that these two genera likely occupy a similar niche, with the *Flavobacterium* proliferating in place of the *Cytophaga* following the spike. *Thiobacillus* is a member of the Betaproteobacteria. This class doubled in the post-spike sample compared to pre-spike, however *Thiobacillus* dropped slightly in post-spike samples (from 1% to 0.87%), and then appears to have grown during the post-spike flush (1.74%) (Fig. 5). Thus the class-level proliferation of Betaproteobacteria cannot be attributed to *Thiobacillus*. At all three time points, there were between 10 and 12% of the sequences assigned to these potential denitrifiers.

Overlaying the sequencing results, the nitrate and nitrite results it appears that antibiotic exposure did not eliminate the denitrifying bacteria, and that function was restored once the drug spike was flushed from the column. As with any sequencing data of this type, presence of the sequence does not indicate expression of functional genes, which in this instance were not expressed during the exposure. It is possible that the observed bacterial changes explain the stimulation of denitrification reported here, and this hypothesis deserves further study. The current experimental design, however, does not allow us to distinguish correlation and causation for the observed relationships. Instead, it gives us an overview of the temporal dynamics of the microbial communities at multiple depths, in response to respiratory conditions and drug exposure.

Together, these results help characterize the spatial and temporal dynamics of bacterial communities in packed soil columns exposed to antibiotics. The columns were packed with the same source material at the top and bottom of the column, allowing us to confirm that the selective pressures due to depth can enrich for different bacterial communities over time. In general, the changes we observed by depth were minor, with the overall percent of reads assigned at the class-level remaining similar. In addition to impact of depth, our results reveal a clear change in the bacterial communities in response to a seven day mixed antibiotic exposure, including changes in potential denitrifying bacteria.

There are important limitations to the current study. With only a

	14d	21d	54d
Cytophaga	1.70	1.35	1.47
Sinorhizobium	1.34	1.45	1.06
Hyphomicrobium	0.93	1.43	1.38
Thiobacillus	1.00	0.87	1.74
Flavobacterium	0.78	0.86	1.73
Azoarcus	0.94	0.74	1.03
Bacillus	0.70	0.55	0.53
Sphingobacterium	0.48	0.58	0.79
Rhodoplanes	0.57	0.58	0.69
Paracoccus	0.24	0.30	0.23
Pseudomonas	0.38	0.23	0.31
Beggiatoa	0.20	0.24	0.21
Nitrobacter	0.26	0.18	0.17
Rhodopseudomonas	0.14	0.14	0.16
Rhodobacter	0.08	0.09	0.20
Azospirillum	0.14	0.14	0.11
Zavarzinella	0.06	0.06	0.09
Bradyrhizobium	0.07	0.04	0.08
Flexibacter	0.05	0.03	0.16
Ochrobactrum	0.03	0.02	0.01
Alcaligenes	0.02	0.01	0.01
Nitrosomonas	0.01	0.00	0.01
Moraxella	0.01	0.01	0.01
Empedobacter	0.01	0.01	0.01
Campylobacter	0.01	0.01	0.00
Magnetospirillum	0.01	0.01	0.00
Achromobacter	0.00	0.01	0.02
Agrobacterium	0.00	0.01	0.02
Neisseria	0.01	0.00	0.00

Fig. 5. Most frequently occurring denitrifying genera under denitrifying conditions over the course of the 54-day experiment. All values displayed are for soil samples taken from the surface soil (top 1.5 cm). Values are expressed as a percentage of the total genus-level assignments for each sample. For example, examining the day 14 sample, 1.7% of all of the bacteria in that sample were classified as *Cytophaga*, and a total of 10% of the bacteria in that sample belonged to the denitrifying bacteria listed. For each of the three sample collection times, the highest values are represented in red, and the lowest values in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

single sample for each of the conditions, we cannot draw conclusions beyond the specific samples examined in this study. Also, while our referenced list of denitrifying genera includes the major known groups of denitrifiers, it is not comprehensive, and there is variation within genera and species as to the carriage of specific genes associated with denitrification. This experiment does not account for that variation. Nonetheless, this study provides a valuable overview of the dynamics of microbial communities in response to exposure to three commonly used veterinary

antibiotics under three respiratory conditions, at three depths, over 54 days. The current study design did allow for comparison of the potential impacts of individual drug components following exposure, revealing lincomycin as the primary driver of the community changes resulting from the mixture.

4. Conclusions

Among the three antibiotics, monensin was not detected in the effluent and only detected in pore water samples collected in the upper portion of the columns regardless of the different environmental conditions. Lincomycin and sulfamethazine were detected in the effluent throughout the study, but effluent concentrations decreased during transport through the columns. Under aerobic conditions, similar behavior was observed under sterile and non-sterile conditions, suggesting limited impact of biodegradation. These findings, combined with the results from the sampling ports, decreasing in the antibiotics' concentrations throughout the columns, suggested that sorption represented the predominant removal mechanism.

The nitrogen transformation in the soil was highly impacted by the addition of antibiotics. Denitrification was affected in three antibiotic treatments (mixed antibiotic, lincomycin-alone, and sulfamethazine-alone)—nitrate concentrations rapidly decreased below analytical detection limits, while nitrite concentrations increased. After discontinuation and during antibiotic flush-out, nitrate and nitrite concentrations returned to previous levels. The microbial communities present in the soil were also temporary impacted by the presence of antibiotics. A subtle long-term change in microbial community composition in the upper portion of the columns was observed, while transient changes occurred in the lower portion. These results suggest that veterinary antibiotics could interrupt nitrogen cycling in soils receiving wastewater runoff or manure applications with potential longer-term microbial community changes possible at higher antibiotic concentrations.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2018.11.093>.

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