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LETTER

Prolonged exposure to manure from livestock-administered antibiotics decreases ecosystem carbon-use efficiency and alters nitrogen cycling

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Abstract

Microbial communities drive soil ecosystem function but are also susceptible to environmental disturbances. We investigated whether exposure to manure sourced from cattle either administered or not administered antibiotics affected microbially mediated terrestrial ecosystem function. We quantified changes in microbial community composition via amplicon sequencing, and terrestrial elemental cycling via a stable isotope pulse-chase. Exposure to manure from antibiotic-treated cattle caused: (i) changes in microbial community structure; and (ii) alterations in elemental cycling throughout the terrestrial system. This exposure caused changes in fungal : bacterial ratios, as well as changes in bacterial community structure. Additionally, exposure to manure from cattle treated with pirlimycin resulted in an approximate two-fold increase in ecosystem respiration of recently fixed-carbon, and a greater proportion of recently added nitrogen in plant and soil pools compared to the control manure. Manure from antibiotic-treated cattle therefore affects terrestrial ecosystem function via the soil microbiome, causing decreased ecosystem carbon use efficiency, and altered nitrogen cycling.

Keywords

Agroecology, antibiotics, ecosystem function, elemental cycles, microbial ecology, stable isotopes.

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INTRODUCTION

Use of antibiotics is under heightened scrutiny due to the increased prevalence of antibiotic-resistant pathogens (Rubin *et al*. 1999; Klein *et al*. 2007; O'Neill 2016). Antibiotic resistance is a multifaceted problem and although the primary focus is on more stringent use of antibiotics in medical settings, the use of antibiotics in the livestock sector is gaining increased attention (Ghosh & LaPara 2007; Kemper 2008; Chee-Sanford *et al*. 2009; Forsberg *et al*. 2012; Popowska *et al*. 2012; Zhu *et al*. 2013; Jechalke *et al*. 2014; Van Boeckel *et al*. 2015, 2017; Koch *et al*. 2017). In the United States, 80% of antibiotics are used in livestock production, representing approximately 15-million kg of antibiotics annually (Lipsitch *et al*. 2002; FDA 2013); globally livestock antibiotic use is projected to increase by 67% between 2010 and 2030 (Van Boeckel *et al*. 2015). After dosing, 40–90% of antibiotics are excreted by livestock either intact or as a biologically active metabolite (Sarmah *et al*. 2006; Gutiérrez *et al*. 2010; Toth *et al*. 2011). Livestock manure either collects in pastures or is applied to cultivated fields as fertiliser, therefore potentially contributing up to 13-million kg of antibiotics to the environment annually (Sarmah *et al*. 2006; FDA 2013). This widespread antibiotic exposure can affect human health through the spread of antibiotic resistance, and also has the potential to directly affect soil microbial communities and the ecosystem processes they regulate (Roose-Amsaleg & Laverman 2016; Wepking *et al*. 2017; Grenni *et al*. 2018).

The effect of antibiotics is an important consideration because microbial communities are key drivers of ecosystem function. Soil microbial communities play an important role in decomposition and elemental cycling in soils (Zak *et al*. 2006; Schimel & Schaeffer 2012; Philippot *et al*. 2013; Graham *et al*. 2014), and impact the composition and productivity of plant communities (van der Heijden *et al*. 2008) often through beneficial and detrimental symbioses, and plant–microbe competition for nutrients (Bonfante & Anca 2009; Raaijmakers *et al*. 2009; van der Heijden & Wagg 2013; Dessaux *et al*. 2016; Raaijmakers & Mazzola 2016). While it is well known that soil microbes compete and signal via antibiotics (Waksman & Woodruff 1940; Fajardo & Martínez 2008; Martínez 2008), the type and amount of antibiotics that soil microbial communities are exposed to in agroecosystems are often novel and certainly present in amounts far surpassing those found in soils naturally (Aust *et al*. 2008; Chee-Sanford *et al*. 2009).

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Evidence is mounting that antibiotics can alter both soil microbial composition through selection by antibiotic pressure, and physiology (Wepking *et al.* 2017) through a stress response (Schimel *et al.* 2007) with the potential to affect ecosystem function. For instance, in settings with known exposure to antibiotics, microbial efficiency has been shown to decrease, as evidenced by increased microbial mass-specific respiration with a subsequent increase in the abundance of antibiotic resistance genes (Wepking *et al.* 2017), indicating that the metabolic costs associated with maintaining active antibiotic resistance may reduce microbial efficiency (Geyer *et al.* 2016). Antibiotic exposure has also been shown to increase methane fluxes from manure (Hammer *et al.* 2016). In addition to these carbon (C) cycling effects, antibiotic exposure may also affect nutrient cycling. Because production of microbial biomass is more demanding for nutrients (*e.g.* nitrogen; N), the shift away from biomass production towards metabolic pathways associated with a stress response could reduce microbial nutrient immobilisation, potentially increasing nutrient losses from ecosystems (Schimel *et al.* 2007).

To investigate the potential effects of prolonged exposure to manure from livestock treated with antibiotics (hereon, these effects are referred to as antibiotic effects) on microbial communities and ecosystem functioning, we applied manure from three groups of cattle (those that received the bactericidal antibiotic cephapirin, those that received the bacteriostatic antibiotic pirlimycin, and control cattle receiving no antibiotics) to grassland plots in a common-garden experiment, along with a no-manure control. The relative impacts of antibiotics on soil microbial communities were examined via determination of fungal : bacterial ratio (hereon F : B) and *16S* and ITS metabarcoding (to assess bacterial and fungal community composition, respectively), and on ecosystem processes via a ^{13}C and ^{15}N stable isotope pulse-chase. We expected that manure itself would positively affect plant growth and lead to an increase in soil C pools. However, when manure was sourced from cattle administered antibiotics, we expected a greater loss of C via respiration, as well as, an overall decrease in ecosystem C-use efficiency due to decreased microbial efficiency (specifically bacterial; Wepking *et al.* 2017). Antibiotics are likely to lead to an increase in F : B (Thiele-Bruhn & Beck 2005; Demoling *et al.* 2009). The implications of this on an ecosystem-scale are subject to debate: given the classical understanding of fungal vs. bacterial contribution to biogeochemical processes, we would expect that systems with a higher F : B would retain more C and N (Bardgett & McAlister 1999; Malik *et al.* 2016). Alternatively, recent work has shown that C and N mineralisation are unrelated to the relative dominance of bacteria and fungi (Rousk & Frey 2015). Therefore, outcomes from this experiment could lend support to either theory in light of recent challenges to the classical understanding of fungal vs. bacterial contribution to biogeochemical processes.

MATERIALS AND METHODS

Experimental design

A common garden experiment with a randomised block design (four treatments, $n = 6$) was conducted at Kentland Farm, Blacksburg, VA, USA (37.199490, −80.584659; 547-m elevation; Unison and Braddock cobbly soils; dominant plant cover is grasses, mostly tall fescue, as well as some herbaceous cover including members of the Lamiaceae and Plantaginaceae families). Treatments included three manure additions (manure from cattle given no antibiotics, or manure from cattle given either cephapirin benzathine or pirlimycin hydrochloride) and one control treatment that received no manure. Both antibiotics are commonly used in the prevention of mastitis in dairy cattle, however, they vary in a number of ways including their fate in the environment (Wind *et al.* 2018) and mode of action. Cephapirin benzathine (Molecular weight = 365.4 g mol^{-1}; pKa = 2.2; water solubility = 3430 mg L^{-1}) is bactericidal, damaging the structural integrity of bacterial cell membranes, whereas pirlimycin hydrochloride (Molecular weight = 447.4 g mol^{-1}; pKa = 8.4; water solubility = 64 900 mg L^{-1}) is bacteriostatic, inhibiting protein synthesis. Hereon, we refer to these four treatments as no-manure control (NMC), control manure (Con), cephapirin manure (Ceph) and pirlimycin manure (Pir).

Manure was applied to appropriate treatments at a monthly rate of 648-g-m^{-2} of wet-weight manure starting in October, 2014 until May, 2015 (213 days) – totaling 4536-g of manure m^{-2}. This amount of manure corresponds with the amount of manure expected given a typical dairy cattle stocking density.

For information regarding manure properties, sourcing and within-manure antibiotic quantification see supplementary materials.

Pulse-chase experiment

Field sampling was conducted in May, 2015. In order to determine whether antibiotic use in dairy cattle affects system-wide elemental cycling, a ^{13}C and ^{15}N stable isotope pulse-chase experiment was conducted. The use of ^{13}C allowed for the tracking of recently photosynthesised C through both above- and below-ground C pools. To accomplish ^{13}C-labelling, a 1-m^2 subplot within each treatment plot was covered with a 0.6-m^3 (0.99-m × 0.99-m × 0.61-m) transparent acrylic chamber (Fig. S1). To prevent gas exchange from outside the chamber, the chamber was fitted into a rubber lined wooden base that was trenched 10-cm into the soil. The rubber liner was then adhered to the acrylic glass chamber using silicon grease. Here, $^{13}CO_2$ was introduced into each chamber via gas-tight ports by reacting 1-g sodium carbonate ($Na^{13}CO_3$, 99 atom% ^{13}C, Sigma-Aldrich; CAS number: 9367-48-4; 113-mg of ^{13}C equivalent) with excess hydrochloric acid. Air was circulated within the chambers using a centrally located internal battery-operated fan. Chamber temperature was monitored using an internal thermometer. CO_2 concentrations within the chamber were monitored via a LI-8100 infrared gas analyser (Li-Cor Biosciences, Lincoln, NE). Chambers were removed after CO_2 levels returned to pre-pulse levels. As temperatures in the chambers can be high during mid-day, pulsing was limited to early morning and late afternoon. The amount of ^{13}C fixed by the plant communities was determined by taking foliar clip samples immediately post-pulse.

Following the ^{13}C pulse-labelling, each plot was also labelled with ^{15}N ammonium nitrate ($^{15}NH_4{}^{15}NO_3$; 98

atom%; Sigma-Aldrich; CAS Number: 31432-46-9; 67-mg of ^{15}N equivalent) in order to examine N-dynamics in response to manure and antibiotic treatments. Ammonium nitrate (300-mg in 1-L of DI water) was added evenly to the soil surface of each 1-m^2 plot. The amount of ^{15}N, similar to Fraterrigo *et al.* (2011), was kept low to avoid a fertilisation effect.

Upon completion of pulse-labelling, we destructively harvested 0.05-m^2 subplots within each 1-m^2 plot at 1, 2 and 7-days post-labelling. An additional subplot was harvested from each 1-m^2 experimental plot prior to pulse-labelling in order to determine natural abundance of ^{13}C and ^{15}N. Above-ground plant material from each subplot was harvested by clipping it at the soil surface. Above-ground plant biomass samples were air-dried, weighed and milled for elemental and isotope analyses. The below-ground portion of the subplot was sampled to 10-cm depth; roots and soil were then separated. Root material was initially air-dried and then later washed, air-dried, weighed and milled for elemental and isotopic analyses. Soils were sieved (4.75-mm), homogenised and stored at either −80 °C, 4 °C or air-dried depending on future analyses (see below).

For soils, we determined POM and mineral-associated soil C and N, and soil microbial biomass C and N. POM and mineral-associated C and N was determined on air-dried soil samples (Lal 2001). Microbial biomass C and N were determined following the chloroform fumigation extraction (CFE) procedure outlined in Fierer & Schimel (2002). Briefly, 40 mls of 0.5 M K_2SO_4 was added to one of each 7-g dry mass equivalent soil pair. One of each pair is then exposed to 1-ml of ethanol-free chloroform to lyse microbial cells and accumulate microbial C and N. Samples are capped, and shaken for 4-h. Samples were then allowed to settle before filtration. Microbial biomass was estimated as the difference between the quantity of C and N between the fumigated and un-fumigated samples. Total organic C and N were then calculated for both the fumigated and unfumigated samples using a Vario TOC Cube (Elemtar, Langenselbold, Germany).

$\delta^{13}C$ and $\delta^{15}N$ in above- and below-ground plant biomass, POM and mineral-associated pools were determined using a Costech ECS 4010 Elemental Analyser (Costech Analytical, Valencia, CA, USA) paired with an Thermo Delta Plus Advantage Isotope Ratio Mass Spectrometer (IRMS; Thermo Fisher Scientific™, Waltham, MA, USA).

Prior to each destructive harvest event ecosystem respiration was measured using a LI-8100 Infrared Gas Analyser (Li-Cor Biosciences, Lincoln, NE, USA). Additionally, two 15-ml subsamples of respired air were captured using a gas syringe and air-evacuated exetainers in order to determine the $\delta^{13}C$ of respired CO_2. The first of two subsamples were collected within the first 15-s of a 2-min respiration measurement period, and the second subsample was collected in the final 15-s. Both subsamples were then analysed for $\delta^{13}C$ using a GasBench II IRMS (Thermo Fisher Scientific™, Waltham, MA, USA). Data were then recalculated to account for varying heights of soil collars and adjusted to optimise the r^2 of the respiration trend-line from each 2-min measurement.

The amount of ^{13}C fixed, respired and the amount of both ^{13}C and ^{15}N contained in above- and below-ground pools was derived using standard isotopic mixing models (Fry 2006). The amount of C and N derived from ^{13}C and ^{15}N additions was calculated as atom excess in a given C or N pool. The atom% excess of a given pool was then multiplied by the total C or N in that pool, giving the mass of ^{13}C or ^{15}N label. The proportion of label in a given pool was calculated as the mass of ^{13}C or ^{15}N label divided by the total amount of C or N of that specific pool. Cumulative ecosystem respiration was calculated via integration. See Supplemental Methods for details related to additional soil parameters, microbial catabolic response profiles and microbial community composition measured in conjunction with the pulse-chase experiment.

Statistics and analysis

Data were analysed using linear mixed models ('lme4' package; Bates *et al.* 2016) with treatment as a fixed effect and plot nested within block as a random effect to account for sampling of plots across time. Model selection (additive vs. interactive) was determined by lowest Akaike information criterion (AIC) score (Akaike 1973). Normality of variance was tested using a Wilk–Shapiro test. Data with non-normal variance was either log or square-root transformed. If normality assumptions were still not met, generalised linear models (GLM; 'car' package; Fox *et al.* 2016) were used using the Gamma family and either the inverse or log link function as all data was continuous and positive (variables containing negative values were standardised). Wald chi-squared tests were used to assess model significance for GLMs. Data were analysed using the R statistical platform (R Core Team 2017). Degrees of freedom for linear mixed models were calculated using Satterthwaite approximations.

For all analyses, we consider statistical significance at $P < 0.05$, and marginal significance at $P < 0.10$. However, it should be noted that it has typically been deemed acceptable to consider changes in soil C pools at $P < 0.10$ (Carney *et al.* 2007; Strickland *et al.* 2010), given that soil C is inherently heterogeneous.

RESULTS/DISCUSSION

Antibiotic effects on active and total microbial biomass

Prolonged manure additions, regardless of antibiotic involvement, increased C mineralisation – an estimate of bioavailable soil C – when compared to NMC ($F_{3,15} = 11.8$, $P < 0.001$; Table S1). More surprising was the observation that active microbial biomass – determined via SIR – was differentially affected by the antibiotic status of the manure (again, we are referring to the effect of exposure to manure from cows given an antibiotic, as an antibiotic effect; Fig. S2a; $P < 0.01$). Specifically, the Ceph treatment exhibited greater active microbial biomass in comparison to the other treatments. Similar to the increase in respiration observed for SIR, increased microbial activity was observed across a range of C-substrates for the Ceph treatment in a catabolic response profile (CRP; Fig. S2b). In contrast, we observed a marginally significant treatment effect on total microbial biomass C ($P = 0.07$) and N ($P = 0.08$), primarily driven by a trend towards increased microbial C and N in the Con treatment

(Table S2). This contrast between active and total microbial biomass may suggest physiological changes, specifically greater mass-specific activity for the Ceph treatment, consistent with findings from previous investigations (Wepking *et al.* 2017). As discussed in the corresponding section, the Ceph treatment did not differ from the other antibiotic manure treatment, Pir, in terms of microbial community composition. Therefore, elevated microbial activity could be due to two, non-mutually exclusive, factors: *i*) the increased presence of lysed cellular material from the action of cephapirin, a bactericidal antibiotic, or *ii*) from a stress response of the microbial community, due to the added metabolic cost of maintaining antibiotic resistance (Schimel *et al.* 2007). This stress response is consistent with previous research on cephapirin use on dairy cattle in pasture systems, and has been suggested as a possible cause of altered ecosystem C cycling, through reduced microbial efficiency (Wepking *et al.* 2017).

Antibiotic effects on fungal : bacterial dominance

As antibiotics detrimentally effect bacteria, we assessed F : B via qPCR to determine shifts in fungi and bacteria. Overall, we observed a significant treatment effect for F : B (Fig. 1; $F_{3,63} = 6.497$, $P < 0.001$) as has been previously observed (Thiele-Bruhn & Beck 2005). Fungal counts increased in the soils receiving manure compared to NMC, as well as in Pir compared to Con. NMC had the lowest ratio, indicating that without manure and associated antibiotics the system is relatively more dominated by bacteria. Notably, within the Pir and Ceph manure treatments, the differences in F : B were due to declines in bacteria (i.e. 16S gene abundance), whereas little change in fungi (i.e. ITS abundance) was observed. This increase in F : B in the Pir treatment may be driven by pirlimycin's bacteriostatic mode of action: pirlimycin typically reduces bacterial growth but does not induce cell lysis. Conversely, cephapirin – a bactericidal antibiotic –causes cell lysis. Lysed cells, as suggested above, cause an increase in labile resources that potentially favor bacteria in spite of the direct negative effects of the antibiotic (Hendrix *et al.* 1986; Rousk & Bååth 2007). This potential net positive effect for bacteria under the Ceph compared to the Pir treatment is supported by a pairwise marginally significant increase in *16S* copies (Fig. 1; $P = 0.08$). It is also possible that decreased inhibition of bacteria in the Ceph treatment can be attributed to cephapirin being relatively more easily degraded than pirlimycin. However, evidence of cephapirin's effect on microbial functional properties was in fact observed, therefore degradability is unlikely to be the explanation for the difference in bacterial effects between antibiotic treatments. Compared to NMC, the addition of manure, regardless of antibiotic involvement, increased the abundance of fungi (i.e. ITS copies) in soil ($F_{3,68} = 5.868$, $P < 0.01$), with Con, Ceph and Pir treatments having greater numbers of ITS copies than NMC. Within the manure-addition treatments, Ceph had a marginally lower abundance of fungi compared to Con ($P < 0.10$), this too could be driven by the mode of action related to this antibiotic.

The primary fungal effect appeared to be driven by manure itself, given that all manure additions increased ITS copies compared to NMC. This could be attributed in part to coprophilous fungi, which specialise in the decomposition of faecal matter, previously shown to be elevated in conjunction with manure (Wepking *et al.* 2017). Additionally, the highest counts of *16S* and ITS copies were measured in the Con treatment. This is likely attributed to the influx of manure-derived resources in the absence of antibiotics. Together these results suggest that while manure additions increase F : B, manure from cattle-administered antibiotics tends to lead to even greater increases, primarily driven by decreased bacterial abundance.

Antibiotic effects on microbial community composition

The results of our community composition assessment largely mirrored the results of the F : B analysis. Bacterial communities changed across our treatments (Fig. 2: *pseudo*-$F_{3,23} = 1.15$; $P < 0.05$; note, the random effect 'block' was dropped from this model because it was non-significant), but fungal communities did not (Fig. S3: *pseudo*-$F_{3,23} = 1.01$; $P = 0.18$). The treatment effect on bacteria was largely driven by differences between Con, and both Pir and Ceph. Notably, a marginally significant pairwise difference was observed between Pir and Ceph ($P = 0.064$). NMC did not differ from the other three treatments, in fact, as NMC can be viewed as a baseline, shifts in bacterial community composition from manure exposure were dependent on the antibiotic status of the manure. Additionally, bacterial beta diversity did not differ between treatments (Fig. 2a; *pseudo*-$F_{3,20} = 1.12$; $P = 0.31$), suggesting that the microbial communities in our antibiotic treatments are distinct from control environments,

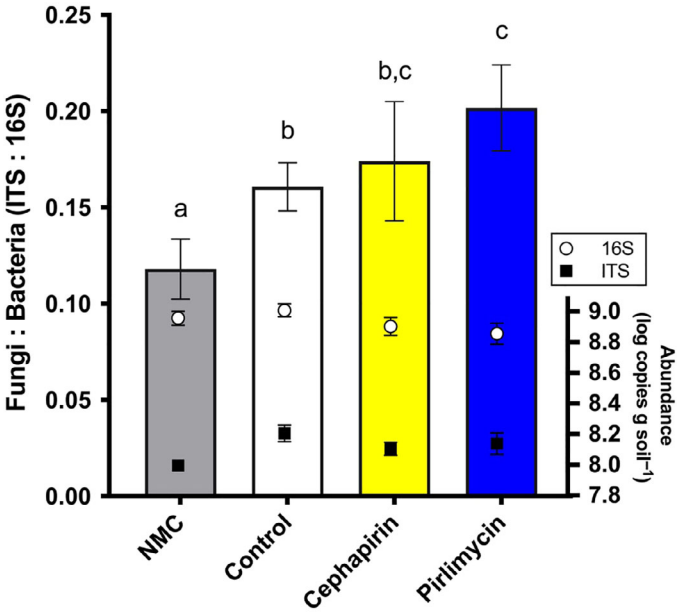

Figure 1 Fungal-to bacterial ratios (F : B) associated with sites receiving manure from cattle administered no antibiotics (Control), administered cephapirin (bactericidal), or pirlimycin (bacteriostatic). Also, shown is the ratio for sites receiving no manure (NMC). Bars represent the mean ± 1 SEM. Letters indicate pairwise differences between treatments; *16S* and ITS copies are indicated by open circles and filled squares, respectively.

and not just more variable in composition. While we did not seek to document the impact of the fecal microbiome on the soil microbiome, previous studies have shown that the fecal microbiome can be impacted by antibiotic exposure (Hammer *et al*. 2016). Therefore, further research into the quantification of this effect would be beneficial, especially investigations into interactions between the faecal and soil microbiomes.

To further investigate OTUs that possibly drove treatment differences, we identified potential OTUs of interest via SIMPER that were common across all pairwise treatment comparisons. This resulted in 32 common OTUs of which only 6 exhibited significant differences between treatments (Fig. 2b). Of these 6 OTUs, 4 were associated with Phyla Acidobacteria and γ-Proteobacteria (2 in each), and 2 were associated with the Phyla Bacteroidetes and Verrucomicrobia (1 in each). Interestingly, the two γProteobacteria were associated with families Acinetobacter and Xanthomonadaceae, which are typically associated with the environment but also include members of human health concern (Berendonk *et al*. 2015). In fact, Wepking *et al*. (2017) observed a similar increase in the genus Acinetobacter in response to cattle administered a cephalosporin. Our results add further support to the likely influence of antibiotics on soil community structure, and further support the proposition that inputs of manure from cattle given antibiotics can shift soil microbial communities towards organisms that are related to those of human health concern (Marti *et al*. 2013; Wind *et al*. 2018).

Interestingly, though, several OTUs associated with phyla that we expect to be more oligotrophic in nature (i.e. Acidobacteria, Verrucomicrobia; Bergmann *et al*. 2011; Fierer *et al*. 2002) also exhibited greater relative abundance in Pir and Ceph. Even some taxa in the family Cytophagaceae could be classified as oligotrophs, especially those involved in cellulose degradation (McBride *et al*. 2014). This greater relative abundance of oligotrophic taxa, primarily in Pir, may not be due to an increase in these groups but due to a decrease in other potentially more copiotrophic groups. That is in the Pir treatment there was an observed decrease in *16S* abundance, suggesting a decline in overall bacterial abundance. Such a decrease, if driven by the antibiotic pirlimycin may have been disproportionate because the antibiotic is bacteriostatic and, as such is likely to have a more detrimental effect on active bacteria (Ding & He 2010; Lobritz *et al*. 2015). Overall, these results suggest that manure from cattle given antibiotics vs. those not, has the potential to lead to shifts in soil bacterial community composition and F : B dominance in a relatively short time (i.e. ~8 months) with implications on microbially mediated ecosystem function.

Antibiotic effects on carbon and nitrogen dynamics

Few differences were observed in most pools of recently fixed C (Table S4), and in the amount of ^{13}C fixed across manure-amended treatments (NMC fixed more ^{13}C relative to total plant C, likely due to the lower plant biomass and identical amount of labelled C added to the chamber; $F_{3,20} = 3.07$, $P = 0.05$). However, we did observe a significant effect of both treatment ($\chi^2 = 18.52$, d.f. = 3, $P < 0.001$), and time ($\chi^2 = 87.18$, d.f. = 2, $P < 0.001$), as well as a treatment × time interaction ($\chi^2 = 41.13$, d.f. = 6, $P < 0.001$), when examining

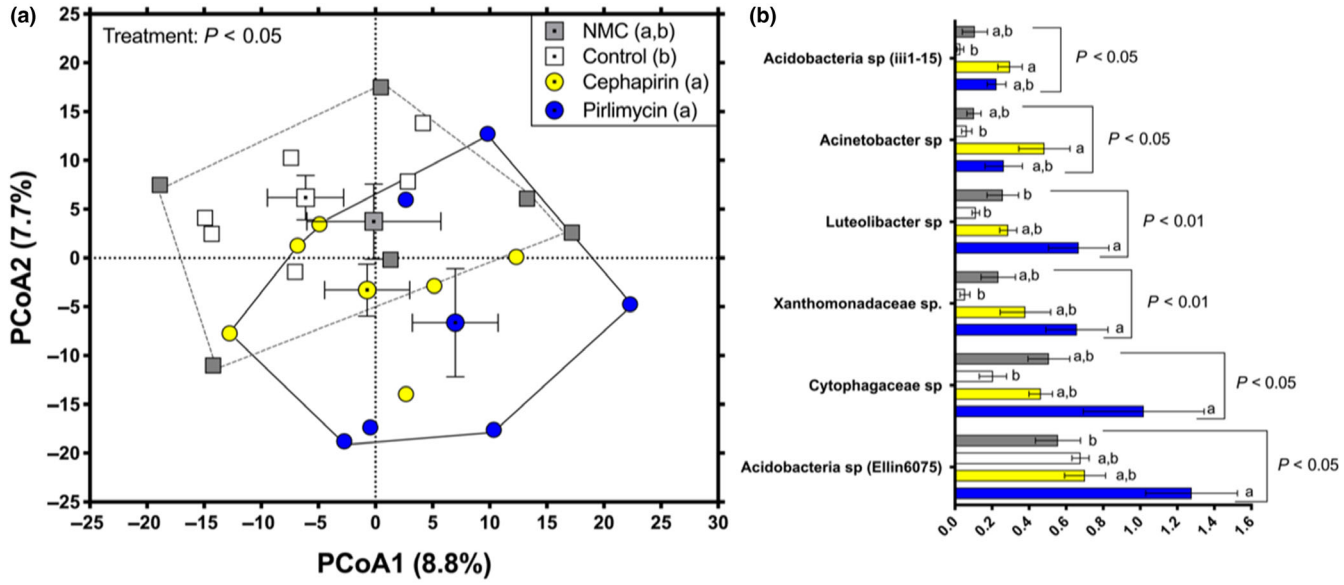

Figure 2 Effect of manure treatments on soil prokaryotic community composition. (a) Principal components analysis showing prokaryote community composition associated with the following treatments: Soil amended with no manure (NMC), soil amended with manure from cattle given no antibiotics (Control), and soil amended with manure from cattle given either a bactericidal antibiotic (Cephapirin) or a bacteriostatic antibiotic (Pirlimycin). Centroids are indicated as symbols with central points and shown as the mean ± 1 SE. Significant pairwise differences between centroids are denoted by different letters in the key. Additionally, lines connecting points indicate those treatments receiving antibiotics (solid line) vs. those that did not receive antibiotics (dashed line). (b) Relative abundance of OTUs that both contributed to dissimilarity between treatments (as determined via similarity percentages) and were statistically significant. Overall treatment statistical significance is indicated by *P*-values, and significant pairwise differences for within OTU comparisons are denoted by different letters.

the ecosystem respiration of recently fixed C (Fig. 2a). Specifically, the Pir treatment exhibited greater initial respiration of ^{13}C compared to the other treatments, but by day 7 of the experiment, respiration of ^{13}C for this treatment was nearly zero (Fig. 3a). The NMC, Pir, and Con treatments exhibited similar respiration dynamics (Fig. 3a). Ecosystem respiration dynamics for the Ceph treatment were more constant during the sampling period compared to the other three treatments (Fig. 3a). A marginally significant difference in cumulative ^{13}C respired across the entire sampling period was explained by the greatest amount of ^{13}C being respired in the Pir treatment with the NMC and Ceph treatments intermediate, and the Con treatment the lowest ($F_{3,15} = 2.72$; $P = 0.08$; Fig. 3a). In fact, nearly twice the amount of newly photosynthesised C was respired – not retained in the soil – in the Pir treatment compared to the Con treatment (Fig. 3a).

These results suggest that manure from cattle administered antibiotics can alter both ecosystem respiration dynamics of recently fixed ^{13}C (i.e. Ceph) as well as the total amount of C respired (i.e. Pir) compared to manure from antibiotic-free cattle. Manure additions from cattle not administered antibiotics may initially suppress respiration slightly compared to sites receiving no manure, possibly driven by decreased plant demand for nutrients. Additionally, if less recently fixed C is lost from a system via respiration, it is likely that more C will be sequestered in that system. This supposition is supported by the significant treatment effect on the proportion of ^{13}C recovered in the mineral pool during the entire experiment (Table S4) with the most ^{13}C recovered in the NMC treatment followed by the Con treatment. Further, at the conclusion of the pulse-chase, we observed a marginally significant treatment effect for ^{13}C found in the mineral-associated soil C pool with the most ^{13}C recovered in the Con treatment among the treatments containing manure (Fig. 3b; $F_{3,15} = 3.04$, $P = 0.06$). Given the slow turnover of the mineral-associated soil C pool (Schlesinger & Lichter 2001), our results suggest that inputs of manure from cattle administered antibiotics may decrease C-sequestration potential. Direct evidence for this potential is the observation of a significant treatment effect for the ratio of fixed ^{13}C to respired ^{13}C (Fig. 3c; $F_{3,15} = 3.65$, $P < 0.05$), an indicator of ecosystem-scale C-use efficiency (Geyer *et al.* 2016). Specifically, we observed that the Pir treatment had the lowest overall C-use efficiency, Con had the greatest, and both NMC and Ceph were intermediate. Soils receiving the Con treatment fixed 2.5-fold more C for every unit of C respired than did the Pir treatment. Together these results indicate that manure inputs from animals-administered antibiotics have the potential to increase C losses from ecosystems compared to manure inputs from animals not administered antibiotics. However, our results also indicate that this effect on C-cycling may be influenced by the specific choice of antibiotics. Further investigation to examine the ecosystem effects of administering an array of antibiotics is merited, especially as agricultural management practices are increasingly seen as opportunities to mediate global climate change (Griscom *et al.* 2017).

A greater proportion of ^{15}N relative to the total N pool was observed with the Pir treatment compared to the other manure-amended plots across all pools sampled (Fig. 4, Table S4), but not necessarily in comparison to NMC. Measuring ^{15}N as a proportion of the total N pool addresses potential difference in plant biomass between treatments (Fraterrigo *et al.* 2011). Within the above-ground biomass ($F_{3,61} = 8.08$, $P < 0.001$; Fig. 4a, Table S4; analysed as the additive model based on the quality of the model using AIC score) and the below-ground biomass ($F_{3,55} = 4.53$, $P < 0.01$; Fig. 4b, Table S4) wherein significantly more ^{15}N was found in the Pir treatment. In addition, a significant and marginally significant time effect was observed in the proportion of ^{15}N in the above-ground and the below-ground biomass, respectively ($F_{2,61} = 6.42$, $P < 0.01$, $F_{2,55} = 2.74$, $P < 0.10$, respectively; Table S4; pooled across treatment). This was characterised by an increased in the proportion of ^{15}N in plant biomass across time. We also observed a significant treatment effect for total N in the above-ground plant biomass ($F_{3,61} = 8.48$, $P < 0.001$; Table S2) and a marginally significant treatment effect for total N in below-ground plant biomass ($F_{3,55} = 2.55$, $P = 0.06$; Table S2; the former was analysed as the additive model, and the latter as an interactive model based on AIC model score). This effect was likely due to greater biomass in the treatments receiving manure vs. NMC (Table S2).

As observed in plant biomass, a greater proportion of ^{15}N in POM (Treatment: $F_{3,61} = 4.77$, $P < 0.005$; Fig. 3c, Table S4) and mineral-associated (Treatment: $F_{3,61} = 2.49$, $P < 0.10$; Fig. 3d, Table S4) soil N pools was observed in the Pir treatment at the conclusion of the experiment compared to the other treatments. The effect of Pir on ^{15}N in the POM N fraction was likely attributable to the root biomass, due to the contribution of plant-derived constituents to this pool (Grandy & Neff 2008). Abundance of ^{15}N in the mineral pool was also increased with Pir, possibly due to decreased plant–microbe competition for N due to an increased F : B in the microbial community. Microbial communities with a higher F : B typically have a higher C : N due to reduced N demand of fungi (Mouginot *et al.* 2014). With the Pir system being more fungally dominated, the overall microbial demand for N is likely lower than for the other three treatments.

We see an increased loss of recently fixed C in the Pir treatment but also an increased uptake of recently added N in this treatment compared to the other treatments. For C, this is particularly unexpected given the increased F : B associated with the Pir treatment. However, the notion that increased F : B leads to a less leaky C-cycle has been called into question (Strickland & Rousk 2010; Rousk & Frey 2015). Rousk & Frey (2015) found that bacterial dominance is linked to a less leaky C-cycle and a less leaky N-cycle, while we observed the potential for greater plant uptake of N. This disparity between our results and those of Rousk and Frey may be because our research was conducted in a grassland system and theirs in a forest. Of particular relevance – among the many differences between these systems – the uptake of available N is likely greater in grasses and forbs during peak growth, over a short period of time, compared to N uptake in trees. When only considering the microbial community, the Pir treatment (i.e. higher F : B) appears to have a leakier N cycle – but when the plant community is also included, this effect is diminished. This highlights the potential for antibiotics to alter plant–microbe interactions and lead to shifts in ecosystem processes.

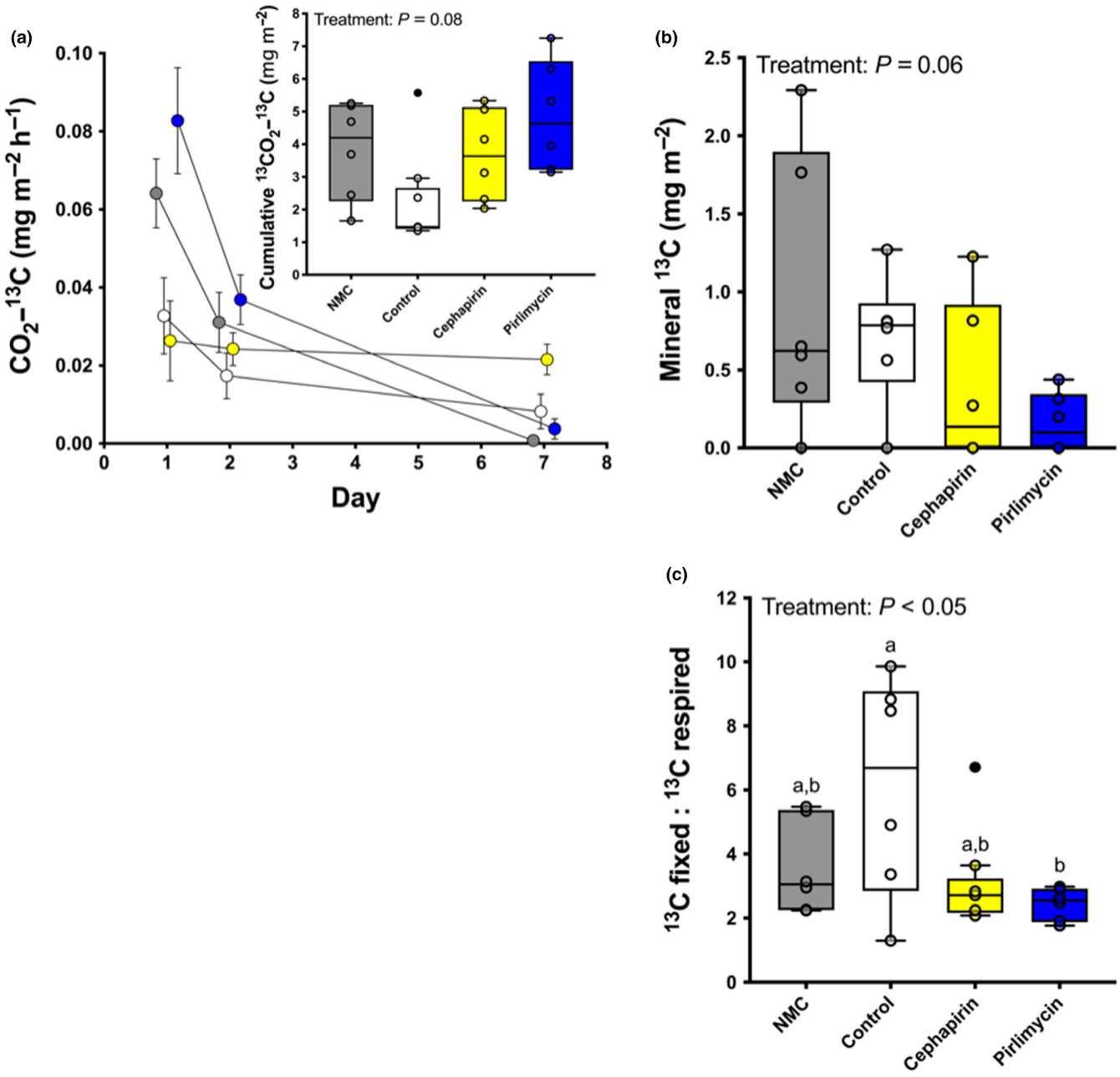

Figure 3 Effect of manure and antibiotic treatments on the cycling of C through the above- and below-ground pools across the following treatments: soil amended with no manure (NMC), soil amended with manure from cattle given no antibiotics (Control), and soil amended with manure from cattle given either a bactericidal antibiotic (Cephapirin) or a bacteriostatic antibiotic (Pirlimycin). (a) Ecosystem respiration dynamics across the 7-day sampling period are shown in the main panel. Points represent the mean ± 1 SE (Treatment: $F_{3,61} = 5.3$, $P < 0.005$; Time: $F_{2,61} = 26.6$, $P < 0.001$). The panel inset shows a boxplot of the cumulative ^{13}C respired across the entire pulse-chase (Treatment: $\chi^2 = 6.86$, d.f. = 3, $P < 0.08$). While the cumulative ^{13}C respired is marginally significant, it represents a doubling of respired CO_2, and is therefore ecologically meaningful. (b) Total accumulation of ^{13}C in the mineral associated soil fraction by the end of the 7-day pulse chase event (Treatment: $F_{3,15} = 3.04$, $P = 0.06$). (c) The ratio of ^{13}C fixed to ^{13}C respired, an indicator of whole ecosystem C-use efficiency (Treatment: $F_{3,15} = 3.65$, $P < 0.05$). Letters indicate pairwise differences between treatments.

Mechanistically, these effects on N dynamics could be due to altered microbe–plant competition for N. In this instance, a bacteriostatic antibiotic – pirlimycin – increased F : B leading to a leakier C cycle, but also a decrease in plant–microbe competition for N (evidenced by increased plant N uptake; 52). Another potential mechanism is reduced competition with mycorrhizal fungi for N with the Pir treatment. Given recent evidence suggesting a C cost associated with N uptake (Shi *et al.* 2016) for mycorrhizal symbionts, if mycorrhizal N uptake increases with a reduction in bacteria then plant N uptake may increase, but more C may be lost from the system. Finally, as this experiment was conducted during peak plant growth and N demand, more N may in fact be lost from the system with decreased plant N demand.

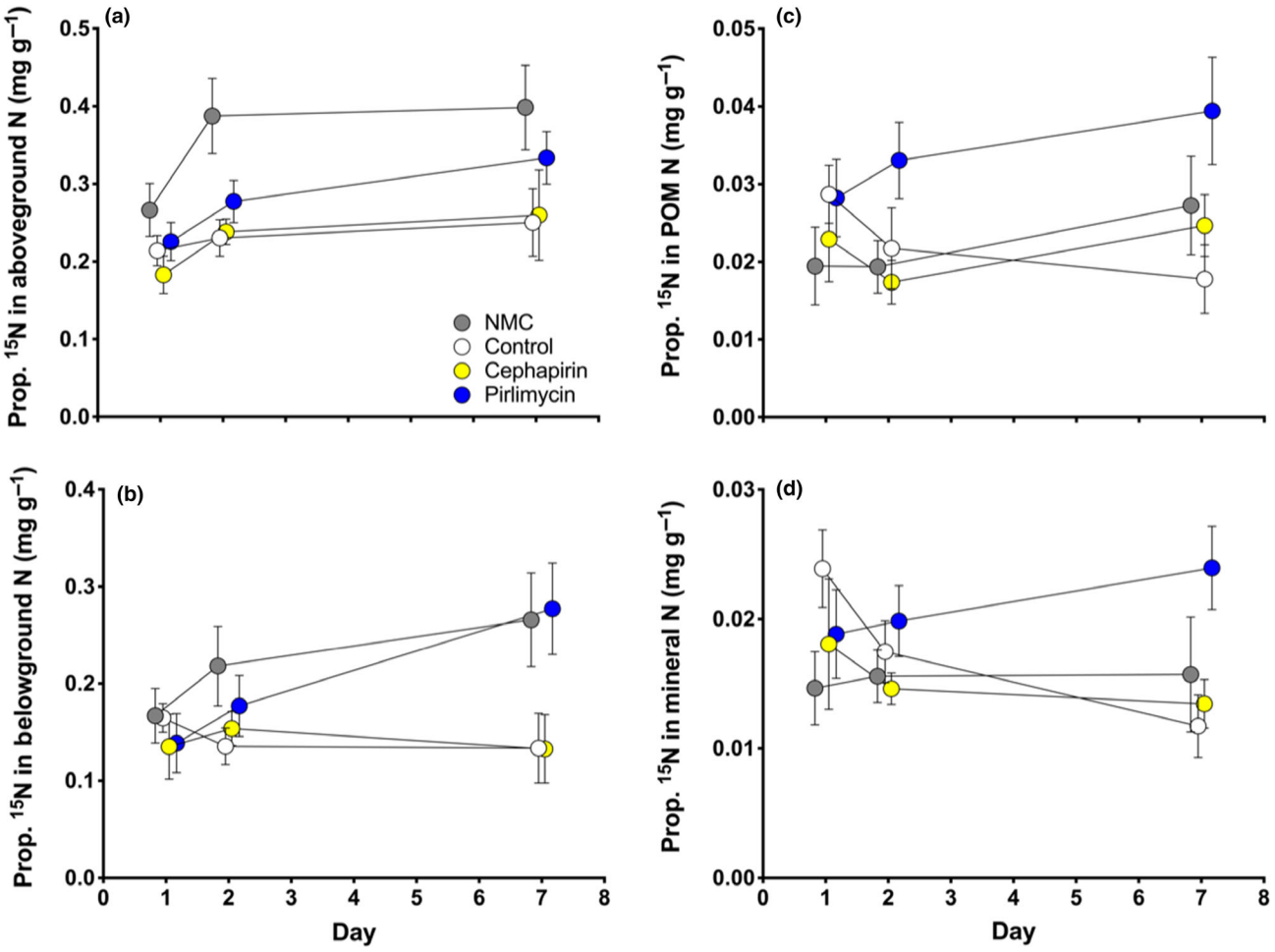

Figure 4 Effect of manure and antibiotic treatments on the cycling of newly added N through the above- and below-ground systems across the following treatments: soil amended with no manure (NMC), soil amended with manure from cattle given no antibiotics (Control), and soil amended with manure from cattle given either a bactericidal antibiotic (Cephapirin) or a bacteriostatic antibiotic (Pirlimycin), and across time. All panels show the proportion of ^{15}N within each respective N pool. Error bars represent ± 1 SEM.

CONCLUSION

Antibiotics affect not just the soil microbiome, but the entire ecosystem; how the ecosystem is affected depends on the antibiotic's mode of action (i.e. bactericidal vs. bacteriostatic). Of the two antibiotics investigated, one in particular – pirlimycin – alters both C and N cycling. This is likely due to changes in microbial composition – as demonstrated by increased F : B, and shifts in bacterial community composition. Increased availability of N appears to occur with decreased C retention in the system – subsequently decreased whole ecosystem C-use efficiency. In contrast, cephapirin increases microbial activity as a stress response – in keeping with previously published research which showed decreased microbial efficiency and increased soil C loss (Wepking *et al.* 2017). While the majority of attention is paid to livestock antibiotic use from the perspective of the proliferation of antibiotic-resistant pathogens and antibiotic resistance genes (Udikovic-Kolic *et al.* 2014; McEachran *et al.* 2015), the impacts on biogeochemical cycling have been overlooked. With global livestock antibiotic use projected to increase by 67% between 2010 and 2030 (Van Boeckel *et al.* 2015) combined with increasing atmospheric CO_2 concentrations, understanding and accounting for the effects antibiotics have on soil microbial communities and whole ecosystem function is imperative.

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AUTHORSHIP

C.W. and M.S.S. designed research. C.W., B.B., J.E.B., K.F.K., K.M., P.P.R., S.S. and M.S.S. performed research. C.W., J.M.L and M.S.S. analysed the data; C.W. and M.S.S. wrote the manuscript; all authors participated in editing and revision of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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