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Exposure to dairy manure leads to greater antibiotic resistance and increased mass-specific respiration in soil microbial communities

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Abstract

Intensifying livestock production to meet the demands of a growing global population coincides with increases in both the administration of veterinary antibiotics and manure inputs to soils. These trends have the potential to increase antibiotic resistance in soil microbial communities. The effect of maintaining increased antibiotic resistance on soil microbial communities and the ecosystem processes they regulate is unknown. We compare soil microbial communities from paired reference and dairy manure-exposed sites across the US. Given that manure exposure has been shown to elicit increased antibiotic resistance in soil microbial communities, we expect that manure-exposed sites will exhibit 1) compositionally different soil microbial communities, with shifts toward taxa known to exhibit resistance; 2) greater abundance of antibiotic resistance genes; and 3) corresponding maintenance of antibiotic resistance would lead to decreased microbial efficiency. We found that bacterial and fungal communities differed between reference and manure-exposed sites. Additionally, β-lactam resistance gene *ampC* was 5.2-fold greater under manure exposure, potentially due to the use of cephalosporin antibiotics in dairy herds. Finally, *ampC* abundance was positively correlated with indicators of microbial stress, and microbial mass-specific respiration, which increased 2.1-fold under manure exposure. These findings demonstrate that the maintenance of antibiotic resistance associated with manure inputs alters soil microbial communities and ecosystem function.

Key Words: Agroecology, soil ecology, ecosystem function

1. Background

Globally, demand for livestock products is increasing [1]. With this demand and subsequent expansion in livestock production, antibiotic use is projected to increase by 67% within the next two decades [2]. Given that in the United States almost 80% of the total antibiotics sold are used in the livestock industry [3, 4] and that 40-95% of the administered antibiotic is excreted in faeces and urine there is the potential to markedly increase antibiotic resistance in soil microbial communities [5-7]. Compounding this probability is the observation that manure from cattle not administered antibiotics can also stimulate an increase in antibiotic resistance in the microbial community [8]. While the human health consequences of both possibilities are being investigated, the effect of manure and/or antibiotic inputs, and increasing antibiotic resistance on soil microbial community composition and ecosystem function are largely unknown, yet potentially important given widespread antibiotic use and projected increased livestock production and subsequently increased inputs of livestock waste [9].

The potential ecological consequences of increased antibiotic exposure and/or maintenance of antibiotic resistance in response to manure inputs on soil microbial communities is largely unexplored. This oversight fails to consider growing evidence that links soil microbial community composition and physiology to ecosystem function [10-13]. Furthermore, microbial efficiency has been tied directly to increased formation of soil organic matter and decreased loss of soil carbon via respiration [14-16]. Observations showing specific antibiotic effects on soil microbial community composition, and physiology [5, 7, 17], thus highlight the potential that the maintenance of antibiotic resistance could ultimately influence ecosystem-scale processes. That is, if soil bacteria must maintain some form of active antibiotic resistance – such as production of β-lactamases – microbial growth efficiency could decrease through increased metabolic

costs, resulting in altered ecosystem function of soil microbes (and likely change in soil microbial community composition). Decreasing microbial efficiency indicated by increased mass-specific respiration could result in subsequent declines in soil carbon (C) retention. This is akin to the widely studied stress response in soil microbial communities (*e.g.* drought), whereby microbes shift allocation of C and nutrients from microbial growth to the production and maintenance of molecules (*e.g.* osmolytes) for survival [18].

To examine the potential implications of the maintenance of antibiotic resistance on ecosystem scale processes we employed a large-scale assessment of reference and manure-exposed soils. We examined how long-term exposure to dairy cattle manure from herds treated with antibiotics can influence, the abundance of antibiotic resistance genes (ARGs) in soil, soil microbial community composition and microbial efficiency. While soils from these 11 paired sites represented a wide variety of edaphic, climate, and biological characteristics, we expected that with prolonged exposure to dairy manure and any excreted antibiotics, the microbial community would be altered. In particular, we expected an increase in the relative abundance of taxa associated with antibiotic resistance in general, and cephalosporins specifically. Secondly, we expected an increase in abundance of ARGs. Specifically, we expected that if antibiotic exposure was an important driver of resistance (as opposed to the manure itself) then this could potentially be indicated by an increase in ARGs related to cephalosporin resistance and little to no change in microbial mass-specific respiration when directly exposed to the cephapirin benzathine – the only antibiotic given to cattle at these sites (personal communication with dairy managers). Finally, we expected that indicators of microbial growth efficiency would decrease with manure and any associated antibiotic exposure due to the increased maintenance demands associated with antibiotic resistance, and

that this would ultimately increase the amount of C respired per unit microbial biomass. This would be apparent as a positive relationship between ARG abundance and mass-specific respiration, even when considering the potential influence of other soil characteristics.

2. Materials and Methods

(a) Study design

Between 21 November 2013 and 1 January 2014 soil samples were collected from 11 dairy farms across the United States (figure S1). At each farm, onsite personnel collected soil samples from areas of cattle congregation (visually assessed and typically located near feed or water troughs, obvious inputs of manure at the time of sampling) and reference sites (a location not heavily trafficked by cattle, within close proximity to the manure-exposed site, free of manure at the time of sampling, but potentially exposed to minimal manure) – hereon, manure-exposed and reference, respectively. Pastures were stocked or had recently been stocked with cattle actively treated with a cephalosporin antibiotic (cephapirin benzathine) prior to the collection of soil samples (personal communication with the individual farm mangers). Cephapirin, an antibiotic used to prevent mastitis, has been shown to be excreted by cattle administered the drug [19]. Three soil samples (0-5 cm depth) were collected per site and combined into one composite sample from each location and then immediately shipped to Virginia Tech, Blacksburg, VA, USA for further processing. Once received, soils were sieved (4 mm), homogenized, and stored at 4°C or -80°C (for determination of ARG abundance and microbial community composition) until further analysis.

(b) Abundance of antibiotic resistance genes and microbial community composition

Microbial community composition was determined for both bacteria and fungi. DNA was

extracted from the soils using MoBio's PowerSoil DNA extraction kit (MoBio Laboratories). Community composition was assessed via amplification of the V4 region of the bacterial/archaeal 16S rRNA gene and the fungal ITS1 region, using primer pairs 515F / 806R, and ITS1 / ITS2, respectively [20]. Amplification followed Caporaso et al. [21]. Amplicons were multiplexed then sequenced on an Illumina MiSeq producing 250bp paired-end reads [21]. Quality filtering and clustering reads into operational taxonomic units (OTUs) were accomplished using USEARCH, following a customized UPARSE pipeline [22]. Taxonomy was assigned to OTUs via the RDP classifier (OTU cut-off for clustering was 97%), using the GreenGenes 13.8 reference database for bacteria/archaea and the UNITE 6.97 database for fungi [23-25]. QIIME was used to generate rarefied OTU tables and alpha diversity estimates [26]. We assessed ARG (*ampC*, *tetO*, *tetW*, and *ermB*) abundance and fungal-to-bacterial ratios— using the ratio of ITS to 16S gene copy numbers—via quantitative PCR (qPCR). The qPCR procedures followed Thames et al. [27] for ARGs and Fierer et al. [28] for fungal-to-bacterial ratios. Our selection of ARGs was based on the following: 1) ARGs confer resistance to various types of antibiotics (*i.e.* bactericidal or bacteriostatic) and are of potential human health concern [29]; 2) we expected that specific ARGs would be affected differently based on manure inputs, antibiotic usage, and/or natural prevalence across our study sites. Specifically, *ampC* (codes for β-lactamase) abundance was hypothesized to be greater with inputs of dairy manure, given that cattle from our study sites are treated with a β-lactam antibiotic (*i.e.* cephapirin) to prevent mastitis; *tetO* and *tetW* (code for Ribosomal protection proteins) may be in high abundance but show no difference between site types, given the overall prevalence of tetracycline resistance in soils; and *ermB* (codes for rRNA adenine N-6-methyltransferase) would be in low abundance and also show no difference between site types, given that erythromycin is only rarely used in dairy management operations [30-32].

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159 *(c) Response of soil communities to antibiotic additions*

160 To assess the potential influence of antibiotic additions on microbial respiration (i.e.
161 active versus simply present), we conducted a 60d laboratory experiment whereby soils
162 from both reference and manure-exposed sites were amended with cephapirin,
163 tetracycline, or erythromycin at a rate of 0.6 mg of antibiotic g dry weight soil^{-1} week^{-1}
164 and then respiration from these soils (*i.e.* CO_2) was compared to respiration from a
165 water-only control. This antibiotic concentration was not intended to mimic field
166 conditions, but instead to maximize the response of the microbial community to a given
167 antibiotic. During this time, we monitored soil respiration via an infrared gas analyser
168 (IRGA; Model LI-7000, Li-Cor Biosciences, Lincoln, Nebraska, USA) using the procedure
169 outlined in Strickland, Callaham [33]. At the end of 60 d, we calculated total mineralized-
170 C via integration and determined both mass-specific respiration (see d below), and the
171 respiratory response ratio as the natural log of the antibiotic treatment divided by the
172 water only control. We expected that lab-based additions of antibiotics (*i.e.* cephapirin,
173 tetracycline, erythromycin) to soils would elicit a greater change in microbial respiration
174 for microbial communities that are naive to these antibiotics (*see* Response of soil
175 communities to antibiotic additions, below, for further details). In contrast, little change in
176 microbial respiration would be expected for additions of antibiotics to soils where the
177 microbial community has had previous exposure, either through direct antibiotic
178 exposure or manure mediated effects. Specifically, we expected that direct cephapirin
179 additions would elicit little change in microbial respiration of manure-exposed soils
180 compared to the change in respiration of reference soils.

181

182 *(d) Microbial stress and soil characteristics*

We determined an array of soil characteristics including soil texture, pH, soil organic C and N in particulate organic matter (POM) and mineral-associated soil fractions, dissolved organic matter C (DOC), microbial biomass C and nitrogen (N), and active microbial biomass via substrate induced respiration (SIR). Soil texture was determined using the hydrometer method [34]. Soil pH was determined in water (1:1 volumetric ratio of water to soil) using a bench-top pH meter (Hatch® sensION+ PH3). Mineral and particulate organic matter (POM) associated C and N were determined by dispersing soils with sodium hexametaphosphate for, at least 18h, and then passing the suspension through a 53 μm sieve. Material >53μm is considered POM material and <53μm is considered mineral-associated material. Concentrations of C and N in these two fractions were determined using a CE Elantech EA 1112 elemental analyser (Thermo Scientific, Waltham, MA, USA). Microbial biomass C and N, and DOC were determined using the simultaneous chloroform fumigation extraction procedure described in Strickland, Devore [35], with N determined colourometrically (Lachat QuikChem® 8500 FIA System) and C determined on a TOC analyser (Ohio Instruments Corporation Model 700). SIR, a measure of active microbial biomass, was determined following Strickland, Devore [35]. Briefly, soil slurries were incubated, after a 1 h pre-incubation with excess substrate (*i.e.* autolyzed yeast extract), for 4 h at 20 C. After the 4-h incubation, SIR is determined via infrared gas analysis of headspace CO_2 concentrations using a static incubation technique. Using the conversion described in Phillips et al. [36] we converted the SIR rate to equivalents of microbial biomass C.

Microbial stress was assessed using two techniques. The first, qCO_2 or the metabolic quotient, was determined according to Wardle and Ghani [37]. Briefly this is a short-term incubation similar to SIR, described above, where each soil is incubated with either water or glucose. qCO_2 is calculated as the ratio of basal respiration (i.e water amended)

to glucose respiration. The expectation is that with increasing microbial stress and/or maintenance demands, qCO_2 will increase. Secondly, we used a 60d soil C mineralization coupled to an average of active microbial biomass determined at the beginning and end of the 60 d period. This estimate allowed us to determine a long-term estimate of microbial mass-specific respiration. As with the short-term qCO_2 estimate, we expected greater respiration per unit microbial biomass to be indicative of greater microbial stress and maintenance demands.

(e) Statistical analyses

The effect of cattle manure inputs on ARG abundance and microbial mass-specific respiration, blocked by site location, was determined via analysis of variance (ANOVA). Relationships between *ampC* abundance and qCO_2 and microbial mass-specific respiration were assessed via regression analysis. Because of the variation across sites and manure input levels (TableS1), we determined the overall importance of *ampC* abundance as a control on microbial stress (*i.e.* qCO_2), via model comparison and selection using an information-theoretic approach [38]. This approach allowed us to compare multiple linear models that included parameters, which we expected would influence microbial stress in soil using Akaike's information criteria for small sample size (AICc) – a metric used to assess model parsimony. These parameters included: *ampC* abundance, silt + clay content, pH, SIR biomass, microbial biomass C:N, POM C:N, mineral-associated C:N, latitude, input level, and the interaction of these parameters with input. These were not randomly determined. For instance, we expected that with increasing silt + clay content that communities would experience less moisture stress and that latitude could be an indicator of temperature stress. Model selection also allows for the determination of 'parameters of interest' via model averaging, allowing for the robust determination of potential controls on microbial stress and in this instance

enabling us to determine if *ampC* abundance is a major control when considering models with a difference in AICc < 4 from the most parsimonious model. Note that models within this AICc range are likely to have substantial empirical support [38]. Additionally, using model averaging for models with a difference in AICc < 4 we determined coefficient estimates.

The effect of manure exposure on bacterial and fungal community composition was assessed via permutational-MANOVA and visualized using principal components analysis. The relationship between bacterial and fungal communities was determined via a Mantel test. To determine which fungal or bacterial taxa contributed to differences between cattle input levels, the percentage contribution of taxa to dissimilarity between inputs was determined. Regression, ANOVA, and multi-model inference were conducted in R [R Core 39] and microbial community analyses were conducted in Primer [40]. When necessary, data were log or square root transformed to meet assumptions of normality and homogeneity.

3. Results and Discussion

(a) Bacterial and Fungal Community Composition

We observed significant differences in bacterial ($F_{1,10}$ = 3.69; P < 0.01) and fungal ($F_{1,10}$ = 3.90; P < 0.01) communities between soils sourced from reference and manure-exposed sites (figure 1A and 1C). For fungal communities (figure 1A and 1B), differences between manure-exposed and reference sites were driven primarily by changes in the relative abundance of genera in the phyla Zygomycota and Ascomycota. The Zygomycota and class Sordariomycetes tended to be in greater abundance in the reference sites (figure 1B). Class Dothideomycetes and phyla Ascomycota were greater in the manure-exposed compared to the reference sites (figure 1B). These shifts in

fungal community composition could be driven by multiple factors including soil C:N ratios, antibiotic inputs, and/or manure additions [41-43]. Interestingly, the relative abundance of genus *Preussia* (class Dothideomycetes) was 3.3-fold greater in the manure-exposed sites (figure S2A). Given that *Preussia* species are generally coprophilous (*i.e.* manure-associated) [44] this provides evidence that *a priori* assessment of manure-exposure and reference locations by onsite personnel was effective. Additionally, we observed a marginally significant, positive relationship between the abundance of the ARG (antibiotic resistance gene), *ampC*, and *Preussia* abundance for the manure-exposed sites ($F_{1,9}$ = 5.09; P = 0.05; r^2 = 0.36; Figure S2B). This relationship may reflect a proxy of manure inputs and associated inputs of the antibiotic cephapirin benzathine, especially given no relationships associated with the other three ARGs. On the other hand, coprophilous fungi are known antimicrobial producers [45], and the positive association with *ampC* abundance found here with *Preussia* (figure S2B) may be indicative of microbial competition. This increase in microbial competition, particularly fungal-bacterial competition, may explain the observations (*i.e.* ARG abundance increases due to manure inputs from cows receiving no antibiotics) of Udikovic-Kolic et al. [8] and is in line with the observation of Fierer et al. [46] showing increased ARG abundance (and microbial competition) associated with more copiotrophic environments. While the exact mechanism causing an increase in ARG abundance requires more attention (*i.e.* competition induced by manure inputs versus direct antibiotic exposure), we would still expect increasing antibiotic resistance with manure exposure to be associated with a decrease in microbial growth efficiency.

For bacterial communities (figure 1C and 1D), the relative abundance of the phylum Firmicutes and class γ -Proteobacteria were ~67 and 70% greater, respectively, in

manure exposed soils (figure 1D). This is notable, given that these two groups are considered indicators of ARGs in the environment [29]. Additionally, greater dissimilarity between reference and manure-exposed bacterial communities was associated with a greater relative increase in total ARG abundance (*i.e.* the sum of the four ARGs measured in this study; $F_{1,9} = 8.14$; $P < 0.05$; $r^2 = 0.48$; figure S3A). This relationship is likely driven by a similar observation for the change in Firmicute abundance from reference to manure-exposed sites ($F_{1,9} = 13.56$; $P < 0.01$; $r^2 = 0.60$; figure S3B), potentially corroborating that Firmicutes are indicators of ARGs. Furthermore, changes in the genus *Acinetobacter* – commonly occurring in soil, water, and on human skin [47] – accounted for 1.31% of the percentage dissimilarity (determined by the contribution of each bacterial genus to the dissimilarity between reference and manure-exposed communities [40]) between reference and manure-exposed sites, with a 25-fold increase in relative abundance of this genus in soils from manure-exposed versus reference sites. This genus contains species associated with low-virulence hospital-associated infections that are of growing human health concern [48-50]. *Acinetobacter* are also known to produce a variety of cephalosporinases and show widespread resistance to β-lactam antibiotics [51]. This suggests that manure from dairy cattle administered cephapirin benzathine as a disease prevention therapy may contribute to a shift in soil bacterial community composition. Inputs of manure from cattle treated with antibiotics may therefore fundamentally alter soil microbial community structure, which in turn likely leads to changes in ecosystem processes [11, 52].

(b) Manure Inputs Increase ARG Abundance and Alter Microbial Respiration in Response to Experimental Antibiotic Additions

We assessed the absolute abundance of four different genes related to β-lactam (*ampC*), tetracycline (*tetO*, *tetW*), and macrolide (*ermB*) antibiotic resistance in soil

312 samples from all sites. Of the ARGs assessed, the average abundance of both *ampC*
313 ($F_{1,10}$=7.4; P<0.05) and *tetO* ($F_{1,10}$=11.4; P<0.01) were 421 and 3,283% greater,
314 respectively, in manure-exposed soils compared to reference soils (figure 2A). This was
315 potentially expected for *ampC,* given the treatment of cattle with cephapirin benzathine,
316 but not for *tetO*, given that farm managers did not report any recent use of tetracyclines.
317 This increase in *tetO* may indicate that manure inputs simply lead to an increase in
318 multiple ARGs. Another, non-mutually exclusive, explanation for this would be co-
319 selection of *ampC* and *tetO*, either because of species selection or because these genes
320 are co-selected on the same plasmid [53]. The observed positive relationship between
321 *ampC* and *tetO* (y=1.57x-2.9; $F_{1,20}$=15.1; P<0.001; r^2=0.43) supports some form of co-
322 selection. Although it is worth noting that while recent use of tetracycline antibiotics at
323 our sites was not reported, we cannot rule out the possibility that this type of antibiotic
324 was used in the past and this could also account for the increased abundance of *tetO*
325 [54].
326
327 In a lab-based experiment, the response of microbial respiration to additions of
328 antibiotics (cephapirin, tetracycline, or erythromycin) was dependent on both the type of
329 antibiotic (*i.e.* bacteriostatic or bactericidal) and whether the soil was exposed to dairy
330 cattle manure. When tetracycline was added to soils, no difference in the respiratory
331 response of microbial communities from the reference and exposed soils was noted
332 (figure 1B; $F_{1,10}$=4.7; P=0.06), even though the abundance of *tetO* was greater in soils
333 exposed to manure. When erythromycin was added to soils, soils sourced from manure-
334 exposed sites exhibited a decreased respiratory response but soils sourced from
335 reference sites exhibited no response to this antibiotic addition (figure 2B; $F_{1,10}$=25.3;
336 P<0.001). This may be due to erythromycin, and bacteriostatic antibiotics in general,
337 having a disproportionate negative effect on metabolic activity in more active microbial

communities [9, 55]. We noted the most marked difference between soils sourced from different sites following cephapirin benzathine application to soils (figure 2B; $F_{1,10}$=56.0; P<0.001). Addition of cephapirin benzathine resulted in an ~2 fold increase in the respiratory response of reference soils versus soils from manure-exposed sites. Together, the combination of greater *ampC* abundance and the less marked respiratory response to cephapirin benzathine additions suggests that communities from the manure-exposed versus reference sites exhibit more pronounced active resistance to cephapirin (figure 2). Together, with inputs of dairy cattle manure and associated antibiotics, we find that *ampC* is in greater abundance and that communities from these sites exhibit less of a response to experimental additions of cephapirin. While the co-occurrence of manure and antibiotics makes parsing out the specific effect of each difficult, these results indicate that the history of antibiotic additions to these soils may be impacting microbial activity. For these reasons and *ampC*'s positive relationship with *tetO*, we focused on relationships between *ampC* and measures of microbial efficiency.

(c) Implications of Manure Inputs and Increased ARG Abundance for Ecosystems

Given that antibiotic resistance – specifically resistance associated with β-lactam antibiotics maintained via the production of β-lactamases – likely increases the maintenance demands of bacteria, thus decreasing microbial efficiency, we examined the stress response of soil microbial communities [qCO2; 56] from the reference and manure-exposed sites. We expected that with increasing *ampC* abundance (a representative β-lactamase gene), a parallel increase in qCO_2 would be observed and that this relationship would be more pronounced in the manure-exposed sites, given that this gene is actively expressed (figure 2). We found no relationship between *ampC* abundance and qCO_2 for reference soils (figure 3; $F_{1,9}$=2.6; P=0.14; r^2=0.22) but a positive relationship was observed for soils exposed to cattle manure inputs (figure 3;

364 $F_{1,9}$=11.83; P<0.01; r^2=0.57). This relationship between qCO_2 and *ampC* abundance in
365 the manure-exposed sites indicates that the maintenance of antibiotic resistance in
366 these communities imposes higher metabolic maintenance costs for soil microbial
367 communities.
368
369 To investigate this physiological response further, we used multi-model inference [38] to
370 assess the overall importance of *ampC* abundance compared to other potential
371 independent variables likely to influence qCO_2 (Supplementary Material). We found via
372 model averaging that *ampC* abundance was the most important independent variable of
373 interest followed by soil texture (table S2; table S3; figure S4). The significance of soil
374 texture may be due to its relationship to soil moisture content, and other edaphic
375 properties (table S3; figure S5). At reference sites *ampC* abundance is relatively
376 unimportant. Instead, with fewer antibiotic additions in the reference sites, soil texture is
377 a stronger predictor of qCO_2 ($F_{1,9}$ = 11.75; P<0.01; r^2 = 0.57; figure S5). Thus, antibiotic
378 inputs may supersede the importance of particular edaphic variables as they relate to
379 ecosystem processes and microbial stress. One interpretation is that with manure inputs
380 from cattle treated with cephapirin benzathine, bacteria up-regulate the production of β-
381 lactamases (figure 2). It is worth noting that for other types of antibiotics, particularly
382 bacteriostatic antibiotics, this increased stress response may not occur. Yet for
383 bactericidal antibiotics, such as β-lactams, this should result in greater maintenance
384 costs for these communities and increased respiratory demand concomitant with active
385 *ampC* abundance (figure 3).
386
387 To determine the broader scale implications of this change in qCO_2 we determined the
388 cumulative amount of soil C respired per unit of microbial biomass (*i.e.* mass-specific
389 respiration) from the manure-exposed and reference sites. On average the manure-

exposed sites respired 2.1 times more C per unit microbial biomass, ranging from as great as a 5.8-fold increase to as low as a 1.1-fold increase (figure 4A – Water treatment –; $F_{1,10}$=20.7; P<0.01). For reference soils, the change in mass-specific respiration was unrelated to *ampC* abundance (figure 4B; $F_{1,11}$=1.8; P=0.21; r^2=0.17) but for soils sourced from manure-exposed sites, mass-specific respiration and *ampC* abundance were positively correlated (figure 4B; $F_{1,11}$=5.8; P<0.05; r^2=0.39). This relationship was even stronger when considering total ARG abundance (*i.e.* the sum of the four ARGs measured; $F_{1,9}$ = 10.02; P<0.05; r^2 = 0.53; figure S6), which could indicate the more general effect of manure inputs on ARG abundance. This suggests that after accounting for the amount of active biomass, sites exposed to manure from cattle treated with cephapirin benzathine mineralize more C, and the magnitude of this increase is positively related to the abundance of *ampC* as well as total ARG abundance.

Our data suggests that this relationship is likely driven by the maintenance of antibiotic resistance [9]. However, it cannot be overlooked that both manure and soil C were not controlled for as a part of this large-scale observational field study, and further investigation of their respective roles is merited. Elevated abundance of ARGs and antibiotic resistant bacteria have also been observed following amendments of manure from dairy cattle not treated with antibiotics [8]**.** More research directly comparing the effect of manure additions from cattle both treated and untreated with antibiotics will help clarify the mechanism leading to antibiotic resistance in soil microbial communities. Yet, while the specific mechanism may be in question (*i.e.* direct antibiotic effects vs. antibiotic mediated microbial competition), we observed greater ARG abundance, specifically *ampC*, in manure-exposed soils and change in *ampC* abundance was positively related to change in mass-specific respiration. Additionally, lab-based amendments of cephapirin benzathine elicited a similar increase in the mass-specific

416 respiration of the reference soils as was observed between the reference and manure-
417 exposed soils (figure 4A). This significant interaction ($F_{1,30}$ = 4.17; P<0.05; figure 4A)
418 between soil source (*i.e.* manure-exposed and reference) and antibiotic amendment (*i.e.*
419 water and cephapirin benzathine) is likely indicative of a trade-off between antibiotic
420 resistance and efficiency and highlights the influence active resistance has on microbial
421 mass-specific respiration. Finally, we suggest that while total soil C, on average, was
422 only 1.7 fold greater in the manure-exposed versus reference sites (table S1), ranging
423 from a 0.9 fold decrease to a 4.1 fold increase, C in these systems is cycling more
424 rapidly, possibly due to the maintenance of antibiotic resistance.
425
426 **Conclusion**
427 Using a large-scale assessment of 11 sites across the United States, we found evidence
428 that exposure to manure from cattle treated with antibiotics drive changes in soil
429 microbial community composition and ecosystem function. First, *ampC*, a β-lactamase,
430 increased with inputs of manure from cattle treated with cephapirin benzathine. The
431 direct addition of this antibiotic elicited less of a respiratory response in soils sourced
432 from these manure-exposed sites indicating that this gene is active. Second, bacterial
433 community composition at manure-exposed sites was dominated by *Acinetobacter*
434 (class γ-Proteobacteria), a genus of bacteria known for its resistance to cephalosporins.
435 Third, qCO_2 and microbial mass-specific respiration were both positively related to *ampC*
436 abundance in manure-exposed sites. Together, and not unlike the findings of Hammer et
437 al. [17], our findings highlight that manure from cattle treated with antibiotics have the
438 potential to markedly alter microbial community composition and the ecosystem
439 processes that these communities regulate. While future research needs to clearly
440 distinguish the relative contribution of manure and antibiotics on microbial processes, as
441 well as whether bacteriostatic antibiotics elicit the same environmental effect, we find

that the manure from cattle treated with a bactericidal antibiotic may lead to significantly more microbial respiration of soil C. This suggests that the expected increase in manure inputs and/or agriculturally derived antibiotics due to intensifying livestock production not only has human health implications [57] but may also have substantial environmental impacts.

Data accessibility. DNA sequences are available from the Sequence Read Archive (project accession number: SRP071347) and all other metadata are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.9r4v1

Author Contributions. CW participated in data analysis and interpretation, and drafted the manuscript; BA carried out soil and qPCR analyses; BB helped design the study and coordinated qPCR and microbial community analyses; JEB helped design the study; JF carried out qPCR, microbial community, and soil analyses; KFK helped design the study; PPR helped design the study; CS carried out qPCR analyses; MSS conceived and helped design the study, conducted data analysis, and coordinated the study. All authors helped draft the manuscript. All authors gave final approval for publication.

Competing Interests. The authors declare no competing interests.

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figure 1. Fungal and bacterial community composition of soils sourced from reference and manure-exposed (+manure) sites. **A)** Principal components analysis showing fungal community composition associated with reference and manure-exposure. Labels indicate the geographic location (i.e. Site) for each pair of samples. Permutational MANOVA indicated significant differences between reference and manure-exposed soils. **B)** Relative abundance of fungal classes at reference and manure-exposed sites. **C)** Principal components analysis showing bacterial community composition associated with reference and manure-exposure. Labels indicate the geographic location (i.e. Site) for each pair of samples. Permutational MANOVA indicated significant differences between reference and manure-exposed soils. **D)** Relative abundance of bacterial phyla and Proteobacterial classes at reference and manure-exposed sites. Note that the difference between site types was primarily due to an increase in the relative abundance of Firmicutes and γ -Proteobacteria.

figure 2. Antibiotic resistance gene (ARG) abundance and the respiratory response to antibiotic additions of soils sourced from reference and manure-exposed (+manure)

sites. **A)** Abundance of *ampC*, *tetO*, *tetW*, and *ermB* ARGs from reference and manure-exposed sites. ARGs were determined via qPCR. Note that abundance is represented as log gene copies. **B)** The natural log of the respiratory response ratio of soils, at reference and manure-exposed sites, exposed to cephapirin, tetracycline, or erythromycin. Values above zero indicate an increase in respiration versus a control soil (*i.e.* no antibiotic addition) and values less than zero indicate a decrease.

figure 3. Relationship between *ampC* abundance and qCO_2, an indicator of microbial stress. Grey circles indicate sites exposed to cattle manure (+manure) and open squares indicate reference sites. A significant relationship was observed for manure-exposed sites but not for reference sites. Additionally, multi-model inference indicates that *ampC* abundance is an independent variable of high importance when considering microbial stress (Supporting Information).

figure 4. The effect of manure-exposure on respiration per unit microbial biomass compared to reference sites. **A)** Comparison of respiration per unit microbial biomass (*i.e.* mass-specific respiration) for manure-exposed and reference sites when amended with water or cephapirin benzathine for 60 days. Significant main effects were noted between manure-exposed and reference sites ($F_{1,30}$ = 29.13; P<0.001), as well as, between water and cephapirin treatments ($F_{1,30}$ = 15.60; P<0.001). We also found a significant interaction between manure exposure and antibiotic amendments ($F_{1,30}$ = 4.17; P<0.05). This interaction was due to no difference in mass-specific respiration between antibiotic treatments for the manure-exposed soils but an increase in mass-specific respiration for the reference soil when treated with cephapirin. Notably the increase in mass-specific respiration from the control to cephapirin treatment we observe for the reference soil is equivalent to what we observe between the reference and

692 manure-exposed soils exposed to water. Letters denote significant pair-wise differences
693 between treatments as determined via Tukey's HSD. Shown are means ± 1S.E. **B)**
694 Mass-specific respiration was positively related to *ampC* abundance under manure-
695 exposed but not for reference sites.

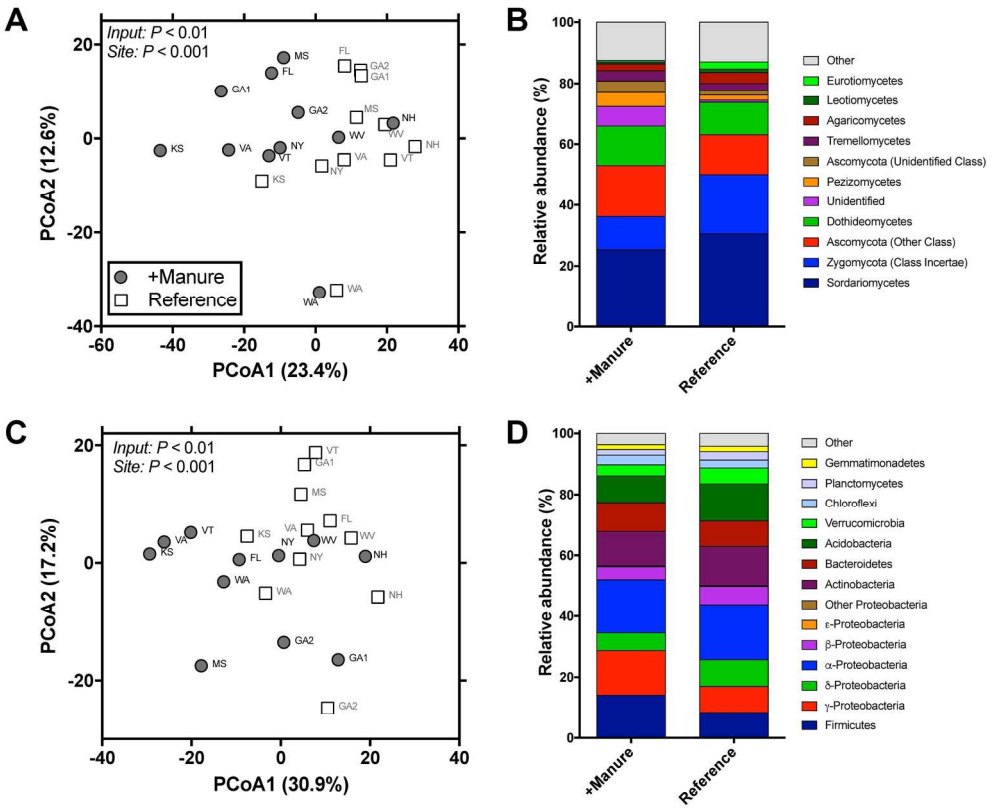

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figure 1
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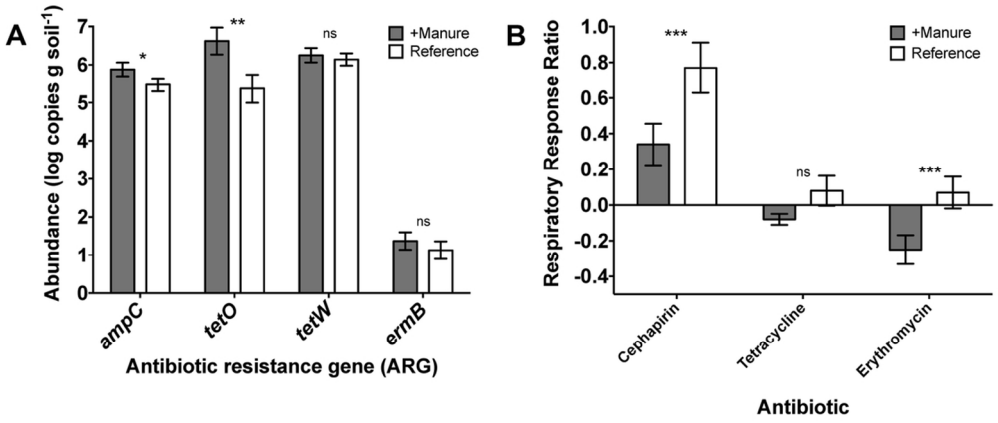

figure 2. Antibiotic resistance gene (ARG) abundance and the respiratory response to antibiotic additions of soils sourced from reference and manure-exposed (+manure) sites. A) Abundance of ampC, tetO, tetW, and ermB ARGs from reference and manure-exposed sites. ARGs were determined via qPCR. Note that abundance is represented as log gene copies. B) The natural log of the respiratory response ratio of soils, at reference and manure-exposed sites, exposed to cephapirin, tetracycline, or erythromycin. Values above zero indicate an increase in respiration versus a control soil (i.e. no antibiotic addition) and values less than zero indicate a decrease.

figure 2
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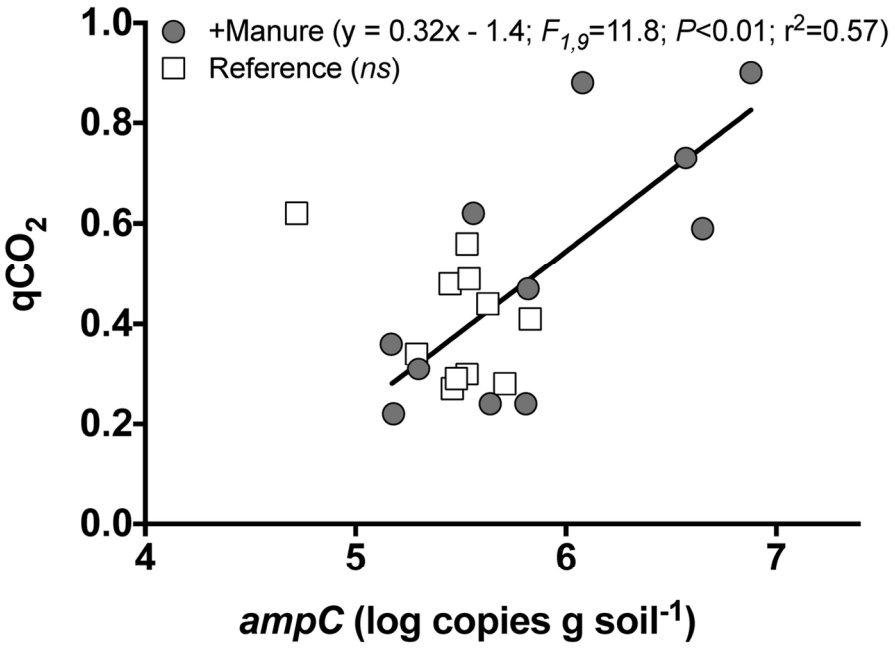

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figure 3
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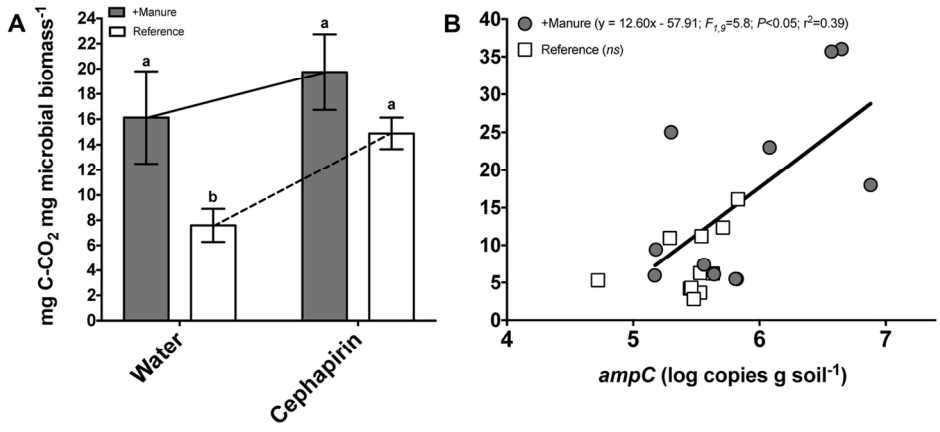

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figure 4
129x58mm (300 x 300 DPI)