

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

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Manuscripts

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

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27 **Abstract**

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

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48 **Key Words: Agroecology, soil ecology, ecosystem function**

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53 1. Background

54 Globally, demand for livestock products is increasing [1]. With this demand and
55 subsequent expansion in livestock production, antibiotic use is projected to increase by
56 67% within the next two decades [2]. Given that in the United States almost 80% of the
57 total antibiotics sold are used in the livestock industry [3, 4] and that 40-95% of the
58 administered antibiotic is excreted in faeces and urine there is the potential to markedly
59 increase antibiotic resistance in soil microbial communities [5-7]. Compounding this
60 probability is the observation that manure from cattle not administered antibiotics can
61 also stimulate an increase in antibiotic resistance in the microbial community [8]. While
62 the human health consequences of both possibilities are being investigated, the effect of
63 manure and/or antibiotic inputs, and increasing antibiotic resistance on soil microbial
64 community composition and ecosystem function are largely unknown, yet potentially
65 important given widespread antibiotic use and projected increased livestock production
66 and subsequently increased inputs of livestock waste [9].

67

68 The potential ecological consequences of increased antibiotic exposure and/or
69 maintenance of antibiotic resistance in response to manure inputs on soil microbial
70 communities is largely unexplored. This oversight fails to consider growing evidence that
71 links soil microbial community composition and physiology to ecosystem function [10-
72 13]. Furthermore, microbial efficiency has been tied directly to increased formation of soil
73 organic matter and decreased loss of soil carbon via respiration [14-16]. Observations
74 showing specific antibiotic effects on soil microbial community composition, and
75 physiology [5, 7, 17], thus highlight the potential that the maintenance of antibiotic
76 resistance could ultimately influence ecosystem-scale processes. That is, if soil bacteria
77 must maintain some form of active antibiotic resistance – such as production of β -
78 lactamases – microbial growth efficiency could decrease through increased metabolic

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

86

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

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

109

110 **2. Materials and Methods**

111 *(a) Study design*

112 Between 21 November 2013 and 1 January 2014 soil samples were collected from 11
113 dairy farms across the United States (figure S1). At each farm, onsite personnel
114 collected soil samples from areas of cattle congregation (visually assessed and typically
115 located near feed or water troughs, obvious inputs of manure at the time of sampling)
116 and reference sites (a location not heavily trafficked by cattle, within close proximity to
117 the manure-exposed site, free of manure at the time of sampling, but potentially exposed
118 to minimal manure) – hereon, manure-exposed and reference, respectively. Pastures
119 were stocked or had recently been stocked with cattle actively treated with a
120 cephalosporin antibiotic (cephapirin benzathine) prior to the collection of soil samples
121 (personal communication with the individual farm managers). Cephapirin, an antibiotic
122 used to prevent mastitis, has been shown to be excreted by cattle administered the drug
123 [19]. Three soil samples (0-5 cm depth) were collected per site and combined into one
124 composite sample from each location and then immediately shipped to Virginia Tech,
125 Blacksburg, VA, USA for further processing. Once received, soils were sieved (4 mm),
126 homogenized, and stored at 4°C or -80°C (for determination of ARG abundance and
127 microbial community composition) until further analysis.

128

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

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

131 extracted from the soils using MoBio's PowerSoil DNA extraction kit (MoBio
132 Laboratories). Community composition was assessed via amplification of the V4 region
133 of the bacterial/archaeal 16S rRNA gene and the fungal ITS1 region, using primer pairs
134 515F / 806R, and ITS1 / ITS2, respectively [20]. Amplification followed Caporaso et al.
135 [21]. Amplicons were multiplexed then sequenced on an Illumina MiSeq producing
136 250bp paired-end reads [21]. Quality filtering and clustering reads into operational
137 taxonomic units (OTUs) were accomplished using USEARCH, following a customized
138 UPARSE pipeline [22]. Taxonomy was assigned to OTUs via the RDP classifier (OTU
139 cut-off for clustering was 97%), using the GreenGenes 13.8 reference database for
140 bacteria/archaea and the UNITE 6.97 database for fungi [23-25]. QIIME was used to
141 generate rarefied OTU tables and alpha diversity estimates [26]. We assessed ARG
142 (*ampC*, *tetO*, *tetW*, and *ermB*) abundance and fungal-to-bacterial ratios— using the ratio
143 of ITS to 16S gene copy numbers—via quantitative PCR (qPCR). The qPCR procedures
144 followed Thames et al. [27] for ARGs and Fierer et al. [28] for fungal-to-bacterial ratios.
145 Our selection of ARGs was based on the following: 1) ARGs confer resistance to various
146 types of antibiotics (*i.e.* bactericidal or bacteriostatic) and are of potential human health
147 concern [29]; 2) we expected that specific ARGs would be affected differently based on
148 manure inputs, antibiotic usage, and/or natural prevalence across our study sites.
149 Specifically, *ampC* (codes for β -lactamase) abundance was hypothesized to be greater
150 with inputs of dairy manure, given that cattle from our study sites are treated with a β -
151 lactam antibiotic (*i.e.* cephalosporin) to prevent mastitis; *tetO* and *tetW* (code for Ribosomal
152 protection proteins) may be in high abundance but show no difference between site
153 types, given the overall prevalence of tetracycline resistance in soils; and *ermB* (codes
154 for rRNA adenine N-6-methyltransferase) would be in low abundance and also show no
155 difference between site types, given that erythromycin is only rarely used in dairy
156 management operations [30-32].

157

158

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 cephalosporin,
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. CO₂) 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, Callahan [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. cephalosporin,
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 cephalosporin
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*

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

204

205 Microbial stress was assessed using two techniques. The first, $q\text{CO}_2$ or the metabolic
206 quotient, was determined according to Wardle and Ghani [37]. Briefly this is a short-term
207 incubation similar to SIR, described above, where each soil is incubated with either
208 water or glucose. $q\text{CO}_2$ is calculated as the ratio of basal respiration (*i.e.* water amended)

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

216

217 *(e) Statistical analyses*

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

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

240

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

250

251 **3. Results and Discussion**

252 *(a) Bacterial and Fungal Community Composition*

253 We observed significant differences in bacterial ($F_{1,10} = 3.69$; $P < 0.01$) and fungal ($F_{1,10}$
254 $= 3.90$; $P < 0.01$) communities between soils sourced from reference and manure-
255 exposed sites (figure 1A and 1C). For fungal communities (figure 1A and 1B),
256 differences between manure-exposed and reference sites were driven primarily by
257 changes in the relative abundance of genera in the phyla Zygomycota and Ascomycota.
258 The Zygomycota and class Sordariomycetes tended to be in greater abundance in the
259 reference sites (figure 1B). Class Dothideomycetes and phyla Ascomycota were greater
260 in the manure-exposed compared to the reference sites (figure 1B). These shifts in

261 fungal community composition could be driven by multiple factors including soil C:N
262 ratios, antibiotic inputs, and/or manure additions [41-43]. Interestingly, the relative
263 abundance of genus *Preussia* (class Dothideomycetes) was 3.3-fold greater in the
264 manure-exposed sites (figure S2A). Given that *Preussia* species are generally
265 coprophilous (*i.e.* manure-associated) [44] this provides evidence that *a priori*
266 assessment of manure-exposure and reference locations by onsite personnel was
267 effective. Additionally, we observed a marginally significant, positive relationship
268 between the abundance of the ARG (antibiotic resistance gene), *ampC*, and *Preussia*
269 abundance for the manure-exposed sites ($F_{1,9} = 5.09$; $P = 0.05$; $r^2 = 0.36$; Figure S2B).
270 This relationship may reflect a proxy of manure inputs and associated inputs of the
271 antibiotic cephalosporin benzathine, especially given no relationships associated with the
272 other three ARGs. On the other hand, coprophilous fungi are known antimicrobial
273 producers [45], and the positive association with *ampC* abundance found here with
274 *Preussia* (figure S2B) may be indicative of microbial competition. This increase in
275 microbial competition, particularly fungal-bacterial competition, may explain the
276 observations (*i.e.* ARG abundance increases due to manure inputs from cows receiving
277 no antibiotics) of Udikovic-Kolic et al. [8] and is in line with the observation of Fierer et al.
278 [46] showing increased ARG abundance (and microbial competition) associated with
279 more copiotrophic environments. While the exact mechanism causing an increase in
280 ARG abundance requires more attention (*i.e.* competition induced by manure inputs
281 versus direct antibiotic exposure), we would still expect increasing antibiotic resistance
282 with manure exposure to be associated with a decrease in microbial growth efficiency.
283
284 For bacterial communities (figure 1C and 1D), the relative abundance of the phylum
285 Firmicutes and class γ -Proteobacteria were ~67 and 70% greater, respectively, in

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

307

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

310 We assessed the absolute abundance of four different genes related to β -lactam
311 (*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 cephalosporin 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 (cephalosporin, 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

338 communities [9, 55]. We noted the most marked difference between soils sourced from
339 different sites following cephapirin benzathine application to soils (figure 2B; $F_{1,10}=56.0$;
340 $P<0.001$). Addition of cephapirin benzathine resulted in an ~2 fold increase in the
341 respiratory response of reference soils versus soils from manure-exposed sites.
342 Together, the combination of greater *ampC* abundance and the less marked respiratory
343 response to cephapirin benzathine additions suggests that communities from the
344 manure-exposed versus reference sites exhibit more pronounced active resistance to
345 cephapirin (figure 2). Together, with inputs of dairy cattle manure and associated
346 antibiotics, we find that *ampC* is in greater abundance and that communities from these
347 sites exhibit less of a response to experimental additions of cephapirin. While the co-
348 occurrence of manure and antibiotics makes parsing out the specific effect of each
349 difficult, these results indicate that the history of antibiotic additions to these soils may be
350 impacting microbial activity. For these reasons and *ampC*'s positive relationship with
351 *tetO*, we focused on relationships between *ampC* and measures of microbial efficiency.

352

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

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

390 exposed sites respired 2.1 times more C per unit microbial biomass, ranging from as
391 great as a 5.8-fold increase to as low as a 1.1-fold increase (figure 4A – Water treatment
392 –; $F_{1,10}=20.7$; $P<0.01$). For reference soils, the change in mass-specific respiration was
393 unrelated to *ampC* abundance (figure 4B; $F_{1,17}=1.8$; $P=0.21$; $r^2=0.17$) but for soils
394 sourced from manure-exposed sites, mass-specific respiration and *ampC* abundance
395 were positively correlated (figure 4B; $F_{1,17}=5.8$; $P<0.05$; $r^2=0.39$). This relationship was
396 even stronger when considering total ARG abundance (*i.e.* the sum of the four ARGs
397 measured; $F_{1,9} = 10.02$; $P<0.05$; $r^2 = 0.53$; figure S6), which could indicate the more
398 general effect of manure inputs on ARG abundance. This suggests that after accounting
399 for the amount of active biomass, sites exposed to manure from cattle treated with
400 cephalosporin benzathine mineralize more C, and the magnitude of this increase is
401 positively related to the abundance of *ampC* as well as total ARG abundance.

402

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

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

447

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

451

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

459

460 **Competing Interests.** The authors declare no competing interests.

461

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465

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472

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649

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

663

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

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

672

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

679

680 **figure 4.** The effect of manure-exposure on respiration per unit microbial biomass
681 compared to reference sites. **A)** Comparison of respiration per unit microbial biomass
682 (*i.e.* mass-specific respiration) for manure-exposed and reference sites when amended
683 with water or cephalosporin benzathine for 60 days. Significant main effects were noted
684 between manure-exposed and reference sites ($F_{1,30} = 29.13$; $P < 0.001$), as well as,
685 between water and cephalosporin treatments ($F_{1,30} = 15.60$; $P < 0.001$). We also found a
686 significant interaction between manure exposure and antibiotic amendments ($F_{1,30} =$
687 4.17 ; $P < 0.05$). This interaction was due to no difference in mass-specific respiration
688 between antibiotic treatments for the manure-exposed soils but an increase in mass-
689 specific respiration for the reference soil when treated with cephalosporin. Notably the
690 increase in mass-specific respiration from the control to cephalosporin treatment we observe
691 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 \pm 1S.E. **B)**
694 Mass-specific respiration was positively related to *ampC* abundance under manure-
695 exposed but not for reference sites.

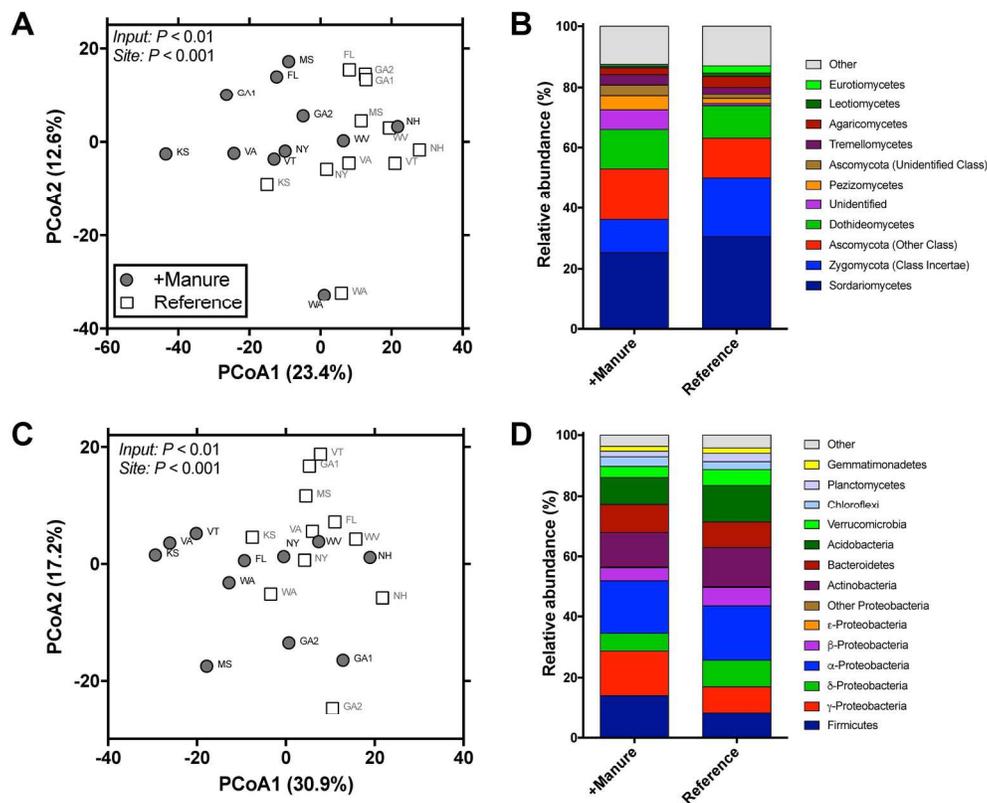


figure 1. Fungal and bacterial community composition of soils sourced from reference and manure-exposed (+manure) sites. A) Principal components analysis showing fungal community composition associated with reference and manure-exposure. Labels indicate the geographic location (i.e. Site) for each pair 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 1
184x153mm (300 x 300 DPI)

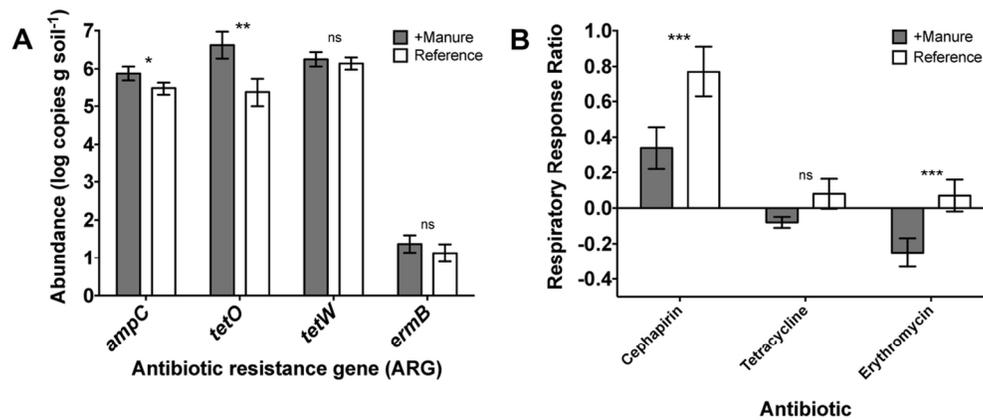


figure 2. Antibiotic resistance gene (ARG) abundance and the respiratory response to antibiotic additions of soils sourced from reference and manure-exposed (+manure) sites. A) Abundance of ampC, tetO, tetW, and ermB ARGs from reference and manure-exposed sites. ARGs were determined via qPCR. Note that 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
107x46mm (300 x 300 DPI)

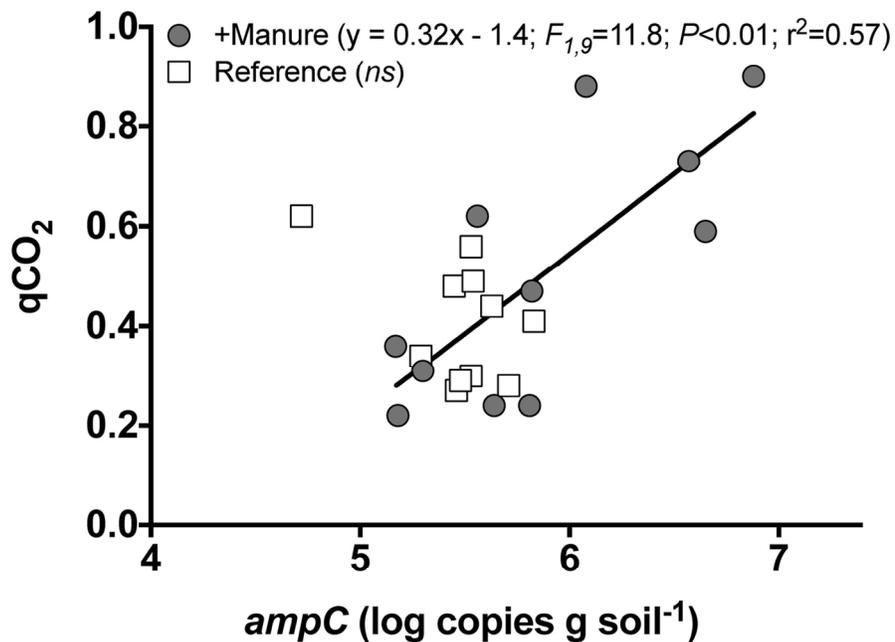


figure 3. Relationship between *ampC* abundance and qCO₂, an indicator of microbial stress. Grey circles indicate sites exposed to cattle manure (+manure) and open squares indicate reference sites. A significant relationship was observed for manure-exposed sites but not for reference sites. Additionally, multi-model inference indicates that *ampC* abundance is an independent variable of high importance when considering microbial stress (Supporting Information).

figure 3
132x93mm (300 x 300 DPI)

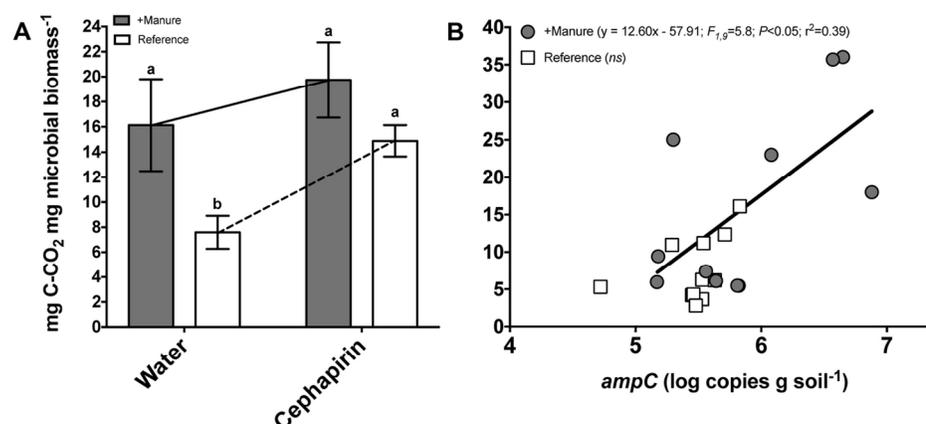


figure 4. The effect of manure-exposure on respiration per unit microbial biomass compared to reference sites. A) Comparison of respiration per unit microbial biomass (i.e. mass-specific respiration) for manure-exposed and reference sites when amended with water or cephalirin benzathine for 60 days. 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 cephalirin 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 cephalirin. Notably the increase in mass-specific respiration from the control to cephalirin treatment we observe for the reference soil is equivalent to what we observe between the reference and manure-exposed soils exposed to water. Letters denote significant pair-wise differences between treatments as determined via Tukey's HSD. Shown are means \pm 1S.E. B) Mass-specific respiration was positively related to ampC abundance under manure-exposed but not for reference sites.

figure 4
129x58mm (300 x 300 DPI)