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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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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   cephapirin 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

5

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 mangers). 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.* cephapirin) 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 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*

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 >53µm is considered POM material and <53µm 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, qCO_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. qCO_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 cephapirin 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 cephapirin

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 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

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 qCO_2 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 qCO_2 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 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-

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; $F_{1,11}$=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; $F_{1,11}$=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   cephapirin 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 cephapirin benzathine elicited a similar increase in the mass-specific

16

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

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

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 cephapirin, 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 cephapirin 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 cephapirin 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 cephapirin. Notably the

690   increase in mass-specific respiration from the control to cephapirin 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 ± 1S.E. **B)**

694   Mass-specific respiration was positively related to *ampC* abundance under manure-

695   exposed but not for reference sites.
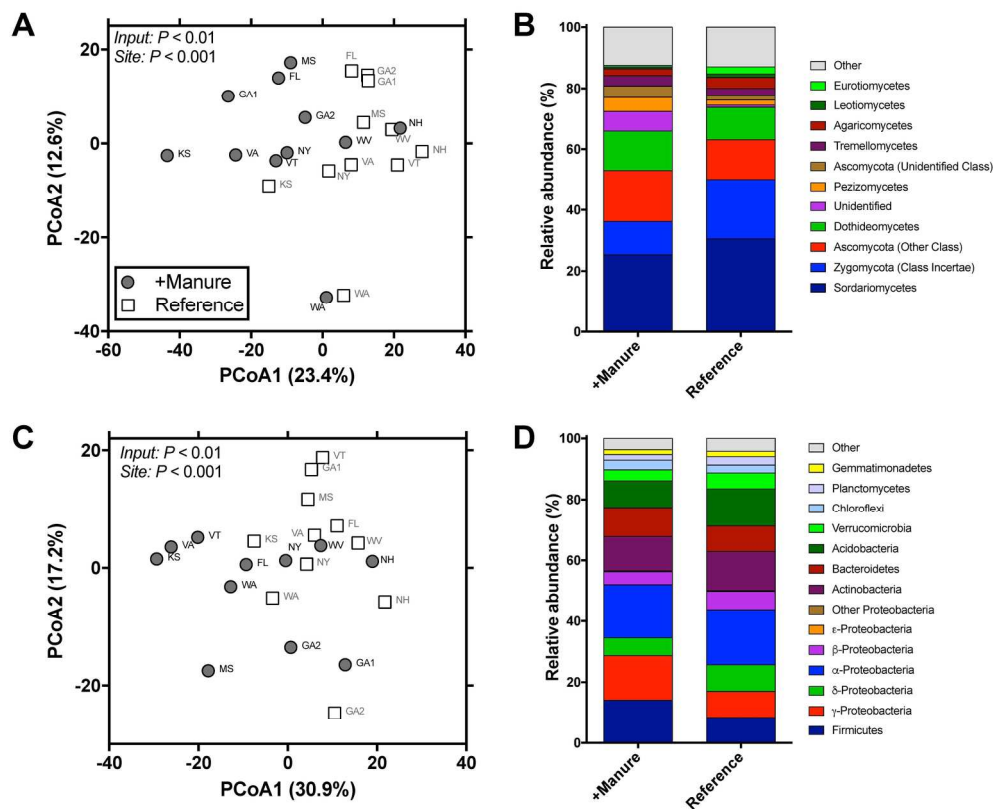
figure 1. Fungal and bacterial community composition of soils sourced from reference and manure-exposed (+manure) sites. A) Principal components analysis showing fungal community composition associated with reference and manure-exposure. Labels indicate the geographic location (i.e. Site) for each pair 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.

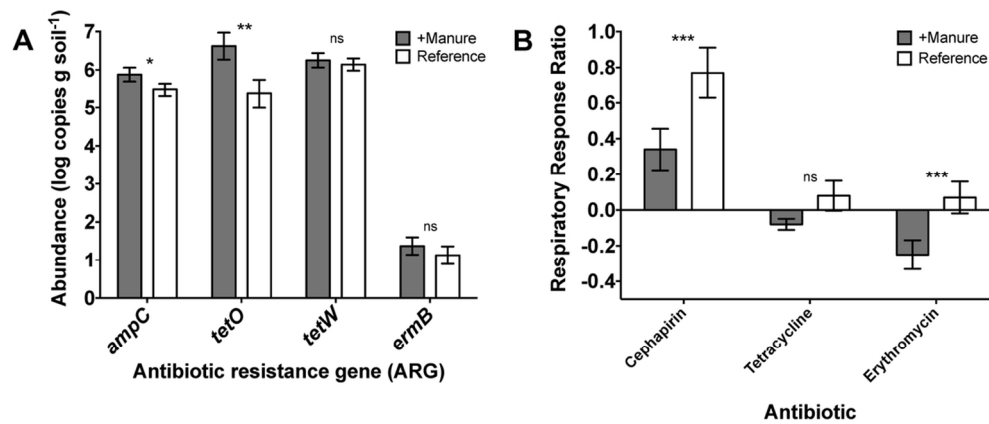figure 1
184x153mm (300 x 300 DPI)

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.
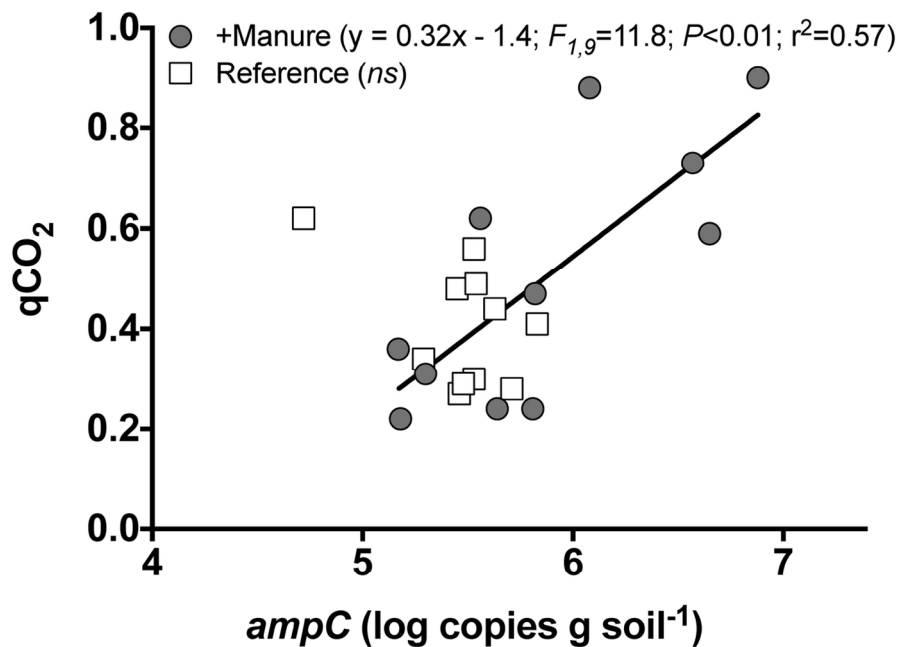
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figure 3. Relationship between ampC abundance and qCO2, an indicator of microbial stress. Grey circles indicate sites exposed to cattle manure (+manure) and open squares indicate reference sites. A significant relationship was observed for manure-exposed 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).
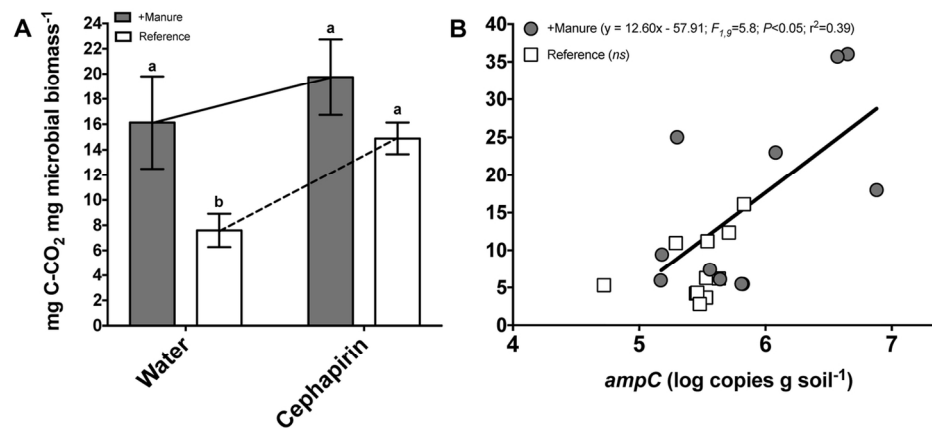
figure 3
132x93mm (300 x 300 DPI)

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 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 manure-exposed soils exposed to water. Letters denote significant pair-wise differences between treatments as determined via Tukey's HSD. Shown are means ± 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)