1 **Title:** Long-term antibiotic exposure in soil is associated with changes in microbial

2 community structure and prevalence of class 1 integrons.

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19 **Running Title:** Antibiotic Impacts in Soil

20 **Abstract**

Antimicrobial resistance is one of the most significant challenges facing the global medical community and can be attributed to the use and misuse of antibiotics. This includes use as growth promoters or for prophylaxis and treatment of bacterial infection in intensively farmed livestock from where antibiotics can enter the environment as residues in manure. We characterised the impact of the long-term application of a mixture of veterinary antibiotics alone (tylosin, sulfamethazine and chlortetracycline) on class 1 integron prevalence and soil microbiota composition. Class 1 integron prevalence increased significantly ($p < 0.005$) from 0.006 % in control samples to 0.064 % in the treated plots. Soil microbiota were analysed using 16S rRNA gene sequencing and revealed significant alterations in composition. Of the 19 significantly different ($p < 0.05$) OTUs identified, 16 were of the Class Proteobacteria and these decreased in abundance relative to the control plots. Only one OTU, of the Class Cyanobacteria, was shown to increase in abundance significantly; a curiosity given the established sensitivity of this Class to antibiotics. We hypothesise that the overrepresentation of Proteobacteria as OTUs that decreased significantly in relative abundance, coupled with the observations of an increase in integron prevalence, may represent a strong selective pressure on these taxa.

Keywords: Antibiotic Resistance, Microbial diversity, 16S rRNA gene, Soil, Class 1 Integrons

40 **Introduction**

41 As a consequence of the global use and misuse of antibiotics, the spread of resistance,
42 particularly through the acquisition of mobile genetic elements by bacterial pathogens, is now
43 seen as one of the most important modern clinical challenges (Arias & Murray, 2009). Given
44 limited development of new and effective treatments it is likely that global infectious disease
45 morbidity and mortality will rise as a consequence (WHO, 2014).

46 The complex and diverse microbiota found in natural environments is increasingly
47 recognised as a potential source of acquired antibiotic resistance. As a route of transmission
48 the extent of human exposure to antibiotic resistant bacteria from the natural environment is
49 not fully understood and is likely to be an underestimated phenomenon (Ashbolt *et al.*, 2013;
50 Finley *et al.*, 2013; Heuer *et al.*, 2011; Wellington *et al.*, 2013). Anthropogenic activities that
51 lead to the introduction of human / animal associated bacteria or antibiotics into the
52 environment may, for example, allow for the exchange and selection of resistance genes
53 between the environmental resistome and clinically important pathogens. One such activity is
54 the application to soil of manures that contain excreted antibiotic residues from medicated
55 animals, a common farming practice in Europe, North America and elsewhere. Within the
56 European Union, the level of antibiotics used per kg of meat product ranges from <20 to 188
57 mg, of which 30 – 90% is excreted in manure (Heuer *et al.*, 2011). Antibiotics have thus been
58 reported at mg kg^{-1} concentrations in liquid manures; tetracycline and sulfamethazine at 66
59 mg kg^{-1} and 40 mg kg^{-1} respectively (Hamscher *et al*., 2001). Sulfamethazine,
60 chlorotetracycline and tylosin, commonly used in commercial pig farming, can reach
61 agricultural soils in this manner (Halling-Sørensen *et al.*, 2005). Several studies have
62 demonstrated a relationship between the application of antibiotic-amended manure and
63 resistance in soil (Heuer *et al.*, 2008; Heuer *et al.*, 2009; Heuer *et al.*, 2011; Heuer & Smalla,
64 2007; Hunde-Rinke *et al.*, 2004; Knapp *et al.*, 2008; Kümmerer, 2004; Wellington *et al.*,

65 2013; Shelver *et al.*, 2010; Halling-Sørensen *et al.*, 2005). These studies have focussed on the
66 changes in abundance of specific resistance genes, such as *sul1*, *sul2* and *tetR*. Here,
67 measurable increase in resistance gene abundance was observed following the application of
68 manure amended with sulfadiazine, tetracycline or oxytetracycline. Byrne-Bailey *et al.* (2009,
69 2011) demonstrated that the addition of faecal slurry from tylosin-fed pigs experimentally
70 amended with sulfachloropyridazine and oxytetracycline to soil resulted in a significantly
71 increased prevalence of class 1 integrons. These genetic elements are associated with
72 transferable resistance gene cassettes to nearly all known antibiotics (Partridge *et al.*, 2009).
73 These elements, commonly identified in bacteria isolated from both human and farm animal
74 populations, and more generally in the environment, have now been proposed as robust
75 markers of anthropogenic pollution (Gillings *et al.*, 2015), and are also likely to be indicative
76 of selection for mobile genetic elements as a consequence of human activity (Gaze *et al.*,
77 2013).

78 Of importance, although much less studied, is the identification of the changes in microbiota
79 arising through exposure to environmental concentrations of antibiotics. This has been
80 previously undertaken using Density Gradient Gel Electrophoresis (DGGE) (Jechalke *et al.*,
81 2014; Kopmann *et al.*, 2013; Reichel *et al.*, 2013; Westergaard *et al.*, 2001), or Terminal
82 Restriction Fragment Length Polymorphism (T-RFLP) (Islas-Espinoza *et al.*, 2012) analysis
83 of fragments of the 16S rRNA gene. Westergaard *et al.* (2001) demonstrated a decrease in
84 diversity (as indicated by reduced banding complexity resolved by DGGE) in soils treated
85 with tylosin as well as an increased abundance of some taxa, the identity of which were not
86 determined. Similarly, Kopmann *et al*. (2013) highlighted only general community changes
87 using DGGE. These approaches suffer from an inherent lack of resolution and depth, and it is
88 not possible to evaluate impacts of antibiotic exposure on rarer taxa. To address these issues,
89 the use of techniques such as 16S rRNA gene sequencing for community diversity analysis,

90 as demonstrated by Ding *et al.* (2014), offer the opportunity to gain deeper insights into soil
91 microbial populations. The use, however, of manure either in addition to or as the source of
92 antibiotic residues makes it clearly difficult to disentangle the direct effects of the antibiotics
93 from those of the microbiota present in the manure itself; a limitation to the Ding *et al.*,
94 (2014) study and those mentioned above.

95 A few studies have evaluated the effects of antibiotics added directly to soil. Islas-Espinoza *et*
96 *al.* (2012) observed an increase in species richness as determined by Shannon-Wiener and
97 Margalef indexes in a soil supplemented with sulfamethazine. Shade *et al.* (2013), using a
98 16S rRNA gene sequencing approach, compared soil bacterial communities under apple trees
99 (*Malus domestica*) that had undergone spray treatments with streptomycin sulphate. No
100 overall effects on community diversity, evenness or structure were observed. However, rarer
101 taxa belonging to Proteobacteria, Bacteroidetes and Actinobacteria, that were not observed in
102 soil samples from under unsprayed trees, were detected in the post-spray samples with one, a
103 *Flavobacterium*, consistently observed (Shade *et al.*, 2013).

104 To characterise *in situ* selection for antibiotic resistance a long-term field study was initiated
105 in 1999 in London Ontario, Canada to evaluate the impact of veterinary antibiotics on soil
106 microbial populations (Topp *et al.*, 2013). This study was designed such that the introduction
107 of bacteria from manure, although a more realistic source of antibiotic residues, is avoided
108 thereby facilitating interpretation of the direct selective effects of antibiotics on soil
109 microbiota. In the present study, the first of its kind to the authors' knowledge, the effects of
110 long-term exposure to a mixture of chlortetracycline, sulfamethazine and tylosin on soil
111 bacterial community composition and abundance of *int1*, a marker for antibiotic resistance,
112 were determined.

113 **Materials and Methods**

114 **Study Site and Sampling**

115 A series of replicated field plots on the Agriculture and Agri-Food Canada research farm in
116 London Ontario received an annual application of veterinary antibiotics to explore potential
117 impacts on soil microbial community composition and antibiotic resistance. Details on the
118 field site, soil management and cropping are provided in Topp *et al.* (2013). Briefly, a series
119 of 2 m^2 plots were established isolated by means of an open fibreglass box (inserted to a
120 depth of ~ 50 cm) with 1 m grassed strips between plots. Soil from this site was characterised
121 as a silt-loam with a pH of 7.4. In June of each year (1999-2004), triplicate microplots
122 received either no antibiotics, or a mixture of tylosin, sulfamethazine and chlortetracycline
123 (commonly used in commercial pig production) calculated to give a soil concentration of
124 1mg each antibiotic kg^{-1} dry weight soil. From 2005, the concentration was increased tenfold
125 to 10 mg kg^{-1} soil. The antibiotics were added as an aqueous solution to the plots by
126 supplementing 1 kg portions of soil sampled from the top 15 cm from each plot with mixtures
127 of each antibiotic, adding the antibiotic-supplemented soil uniformly to the surface of the
128 microplot, and manually tilling this in thoroughly to a depth of 15 cm. Control plots were
129 managed exactly as the antibiotic treated plots, except that no antibiotics were added to the
130 1kg portion of soil taken into the laboratory. Plots were cropped continuously to soybeans
131 (*Glycine max* var. Harosoy) during each growing season, and received no further
132 management other than manual weeding. In the present study, triplicate untreated control
133 plots, and triplicate plots treated with 10 mg kg^{-1} antibiotics were sampled in the summer of
134 2008, ten years after the start of the experiment. Triplicate soil samples, generated by
135 collection and pooling of six 20-cm soil cores and sieved to a maximum particle size of 2
136 mm, were taken from each plot, thus there are a total of nine replicates for the control, and
137 nine for the 10 mg kg^{-1} plots.

138 **DNA Extraction**

139 3.5 g of freshly frozen, stored soil was thawed and underwent DNA extraction using the
140 PowerMax® Soil DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA) according to
141 the manufacturer's instructions.

142 **Class 1 Integron Prevalence**

143 The abundance of class 1 integrons was estimated according to the copy number of class 1
144 integrase genes as described in Gaze *et al.* (2011) Briefly, class 1 integrase and 16S rRNA
145 gene copy numbers were estimated by real-time PCR using Sybr Green chemistry (Applied
146 Biosystems, UK). Molecular prevalence was calculated by dividing the number of integrase
147 genes by the number of 16S rRNA gene copies, with corrections made for 16S rRNA gene (7
148 in *E. coli* in seeded standards, mean 2.5 copies per genome in all bacteria) and IncPβ R751
149 copy number (average 6 copies per cell in seeded standards).

150 **16S rRNA Gene Amplification and 454 Sequencing**

151 Amplification of 16S rRNA genes was done using primers 8F (5'-
152 AGAGTTTGATCCTGGCTCAG-3') and 534R (5'-TIACCGIIICTICTGGCAC-3') (Baker *et*
153 *al.*, 2003) targeting variable regions 1,2 and 3 (V1-3) (ATDBio, Southampton, UK). In
154 addition to Multiplex Identifier Adaptors (MIDs), taken from Roche Applied Sciences
155 technical bulletin TCB-2010-010, 16S rRNA gene PCR primers also included the sequences
156 required for emulsion PCR and sequencing. PCR was done with a GeneAmp® PCR System
157 9700 (Applied Biosystems, UK) instrument using the following conditions: 94°C for 5min,
158 35 cycles of 94 °C, 5s denaturation; 42 °C, 30s annealing, and 72°C, 30 s extension; followed
159 by 72°C, 10min final extension. Each sample was amplified in triplicate 50 µl reactions
160 consisting of primers at final concentrations of 10 µM, $MgCl_2$ at 1.5 and dNTPs at 200 µM.
161 Triplicate PCR reactions for each were pooled and purified using the MinElute® PCR
162 Purification Kit (Qiagen, Crawley, UK) prior to use in emulsion PCR. 454 Sequencing™

163 (Roche Diagnostics Ltd, Germany) was done using GS FLX Titanium chemistry. The raw
164 sequence data have been submitted to the NCBI SRA, accession No. SRP062224.

165 **Quality Filtering, OTU Picking and Taxonomic Assignment**

166 Sequences were processed using the Quantitative Insights in Microbial Ecology (QIIME)
167 pipeline v.1.3.0 (Caporaso *et al.*, 2010). Following splitting by barcode sequence, pre-
168 processing filtering was done using default settings apart from the maximum sequence length
169 which was set at 600 bp to account for amplicon length. Remaining sequences (33.6%) were
170 clustered using uclust (Edgar, 2010) into OTUs with a 0.04 dissimilarity index (96%
171 sequence similarity). This index was previously shown to enable the most accurate
172 reconstruction of microbiome OTU richness and taxonomy using variable regions one, two
173 and three when compared to the full-length 16S rRNA gene (Kim *et al*., 2011). Taxonomic
174 assignments were made using the ribosomal database project (RDP) classifier against the
175 Greengenes 16S rRNA reference OTU database (http://greengenes.lbl.gov) and then aligned
176 using PyNAST (Caporaso *et al.*, 2010). Finally, chimeric sequences (26% of the total aligned
177 sequences) were removed using ChimeraSlayer, the alignment filtered and a phylogenetic
178 tree built by FastTree (Price *et al.*, 2012).

179 **Alpha and Beta Diversity**

180 Observed species richness, Chao1, Phylogenetic Distance and the Shannon index were
181 computed in QIIME. Beta Diversity was determined using the UniFrac distance metric
182 (Lozupone & Knight, 2005), and visualised using principal coordinates analysis (PCA).

183 **Statistical Analysis**

184 Unless stated, significance testing was done using paired t-test in RStudio v 0.98.994, or G-
185 test (log likelihood ratio) with Bonferroni correction within the QIIME package. G-test was
186 used to determine changes in the abundance of OTUs between the untreated and treated soils.

187 **Results**

188 Class 1 integron prevalence in the control samples was 0.006 ± 0.009% compared to 0.064 ±
189 0.056% in the treated samples. This difference was determined to be significant using a chi-
190 square test for comparisons of proportions (from independent samples) $p < 0.005$.

191 A total of 286 126 reads remained after size selection, quality filtering and removal of
192 chimeric sequences. Depth per sample ranged substantially from 3 000 to 66 000. The
193 average sequencing depth for treated and untreated samples was 12 966 and 18 825
194 respectively. The mean depth was 30 119 ±13 362.

195 The dominant bacterial phyla are shown in Figure 1. Proteobacteria represented the most
196 abundant phyla with 32.2 ± 0.03% and 37.5 ± 0.05% of sequences for the treated and
197 untreated samples respectively. Acidobacteria (11.3 ± 0.03% treated; 9.5 ± 0.03% untreated),
198 Actinobacteria (7.2 ± 0.02 % treated; 7.5 ± 0.01 % untreated), Bacteroidetes (8.9 ± 0.01%
199 treated; 11.6 ± 0.04% untreated) and Verrucomicrobia (4.6 ± 0.007 % treated; 4.9 ± 0.01 %
200 untreated) were the other abundant phyla. Comparison of relative abundances in treated and
201 untreated plots is shown in Figure 2. Only the Proteobacteria was shown to significantly
202 decrease in abundance ($p = 0.03264$) in the antibiotic treated samples.

203 At a p value of < 0.05, there were 19 OTUs with an abundance that had altered following
204 antibiotic treatment. Of these, 16 were classified to genus level, with the remainder classified
205 to family level and above (Table 1). Sixteen of the 19 OTUs identified belonged to the
206 Phylum Proteobacteria, with most identified as Betaprotobacteria. Over half of those assigned

207 to Betaproteobacteria were assigned to the Order Burkholderiales with *Aquabacterium*,
208 *Methylibium*, *Pelomonas*, *Pseudorhodoferax*, *Variovorax*, *Duganella* and *Massilia* the
209 identified genera. An additional notable genus identified within the Gammaproteobacteria
210 that was reduced in abundance was *Pseudomonas. Flavobacterium* of the Phylum
211 Bacteroidetes was also identified. All significantly different OTU counts indicated a decrease
212 in abundance in response to antibiotic treatment except for one, belonging to the Class
213 Cyanobacteria, which increased with a p value of 3.23×10^{-7}.

214 A summary of diversity and richness estimates are given in Table S1. The Shannon Index (a
215 measure of species diversity and evenness) was unchanged between treated and untreated
216 samples. Observed species, phylogenetic diversity and Chao1 all indicated reduced diversity
217 in treated samples.

218 Samples were normalised to an even depth of 10 672, prior to beta diversity analysis, to limit
219 potential biases associated with differences in number of sequences per sample. Consequently
220 three samples were excluded; two from a single treated plot (samples seven and nine) and a
221 further sample from an untreated plot (sample 16). Principal components analysis (PCA) of
222 the UniFrac distance metric is shown in Figure 3 and reveals a clear separation between
223 treated and untreated samples.

224 **Discussion**

225 The impact of long-term application of veterinary antibiotics on soil microbial communities
226 was assessed using real-time PCR analysis for class 1 integron prevalence combined with
227 microbial community profiling. We believe this is the first study to determine the effects
228 from the direct application of veterinary antibiotics in the absence of manure with the
229 resolution achievable by16S rRNA gene sequence analysis. We have shown that a decade of
230 annual exposure to a mixture of antibiotics at 10 mg kg^{-1} led to a statistically significant

231 increase in the prevalence of class 1 integrons. Class 1 integrons can be detected in pristine /
232 unpolluted soils and sediments even in the absence of manure treatment (Jechalke *et al.*,
233 2014) have a class 1 integron prevalence of approximately 0.002% (unpublished results, W.
234 H. Gaze, personal communication) (Gaze *et al.*, 2011). The 0.064% observed in treatment
235 plots was higher than that previously observed in soil that had undergone application of
236 antibiotic amended pig slurry (0.01%) (Byrne-Bailey *et al.*, 2011), and soil 12 months after
237 sewage cake application (0.02%) (Gaze *et al.*, 2011) but lower than one month after
238 application (0.36%). Integron prevalence in aquatic sediments has been shown to correlate
239 with specific land uses (Amos *et al.*, 2014), suggesting terrestrial inputs into river catchments
240 and ultimately to receiving coastal waters where human exposure may occur (Leonard *et al.*,
241 2015).

242 Changes in the microbial community profile of treated soils were characterised using 16S
243 rRNA gene (V1-3) sequencing. The decision to analyse samples without pooling prior to
244 either PCR amplification or DNA extraction was made due to the demonstrated negative
245 impact these have on OTU detection (Manter *et al.*, 2010). Ultimately not pooling the
246 samples limited the achievable depth of sequencing per sub-sample as effort was split
247 between 18 individual replicates. It was also hypothesised that sequencing replicate rather
248 than pooled samples would minimise the risk of misinterpreting local spatial variability that
249 has previously been shown to exist and contributing to biogeographical patterns in soil
250 microbial community compositions (Yergeau *et al.*, 2009).

251 Reduced abundances in *Pseudomonas*, Sphingomonadaceae and Families of the Order
252 Burkholderiales (*Burkholderiales incertae sedis*, *Comamonadaceae* and *Oxalobacteraceae*)
253 (Table 1) agree with the observations of Ding *et al.* (2014). As the authors compared
254 applications of manure supplemented with sulfadiazine with applications of unsupplemented

255 manure they concluded that observed differences were attributable to impacts of the
256 antibiotic, and their results are therefore concordant with the findings of the present study.
257 The low number of statistically significant OTUs reported here is a consequence of
258 Bonferroni correction; by nature a conservative method of adjustment that has been
259 highlighted previously to increase the likelihood of type II errors (false negatives) (Perneger,
260 1998). In fact, the separation observed between treatment groups in Figure 3 suggests that the
261 limited number of statistically significant OTUs detailed in Table 1 represents only a fraction
262 of those that have been impacted by the addition of antibiotics. A deeper level of sequencing
263 would almost certainly resolve this and enable further identification of genera that are
264 responsible for these changes. Only one OTU was shown to increase significantly, belonging
265 to the Cyanobacteria (Table 1). This is an unexpected finding given the known sensitivity of
266 members of this Class, which have seen their use in antibiotic environmental toxicity
267 bioassays (Van der Grinten *et al*., 2010).
268 Rising antimicrobial resistance is clearly of global concern and a huge challenge for the
269 medical and scientific community. Determing the impact of anthropogenic activities is a vital
270 aspect of antibiotic stewardship in this regard. This study demonstrates, for the first time, that
271 exposure of soil to veterinary antibiotics causes an increase in the prevalence of genetic
272 elements, class 1 integrons, which can integrate a wide range of antibiotic resistance genes
273 and are implicated in acquired resistance in clinically significant human pathogens. Many
274 class 1 integrons also carry *sul1* in the integron backbone, which would select for the genetic
275 element in the presence of sulfonaimides such as sulfamethazine. Additionally, a measurable
276 shift in the structure of the microbial community was observed. Taxa decreasing in
277 abundance are likely to be under strong selection for acquisition of resistance mechanisms,
278 and class 1 integron carriage has been widely reported within the Proteobacteria; the phyla
279 most affected in the present study. Further study is warranted to elucidate the link between

280 these two reported phenomena. Lastly it should be noted that the 10 mg kg^{-1} concentration

281 used here is at the higher range of what would be expected in a realistic exposure scenario

282 (Aust *et al.*, 2008; Heuer *et al.*, 2008). Therefore further experiments should be undertaken to

283 verify that the present observations hold true under normal farming conditions.

284

285 **Conflict of Interest**

286 None to declare.

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294

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418 Figure 1. Relative abundance, expressed as a percentage, of major bacterial phyla in
419 antibiotic treated (Samples 1 to 9) and untreated soil samples (10 to 18) using V1-3 of the
420 16S rRNA gene.

421 Figure 2. Box and whisker plots (n=9) comparing the relative abundances of each of the
422 dominant phyla between antibiotic treated and untreated plots. T refers to treated, u to
423 untreated. Boxplots show the first and third quartile (bottom and top lines of the box), the
424 median (middle line of the box) and the smallest and largest observations (bottom and top
425 whiskers) of the data distribution. The outside dots indicate the 5th and the 95th percentiles.
426 The average value is indicated by the solid line.

427 Table 1. OTUs that demonstrated a statistically significant (p < 0.05) change in relative
428 abundance. Values indicate the total count for each OTU between treated and untreated plots.
429 *lowest level of taxonomic assignment indicated in parentheses for OTUs that weren't
430 classified to genus level.

431 Figure 3. Principal component analysis showing OTU beta diversity of soils that have
432 undergone antibiotic treatment (blue) compared to untreated (red) based on weighted (A) and
433 unweighted UniFrac (B). Replicate samples of each plot are indicated by shape.

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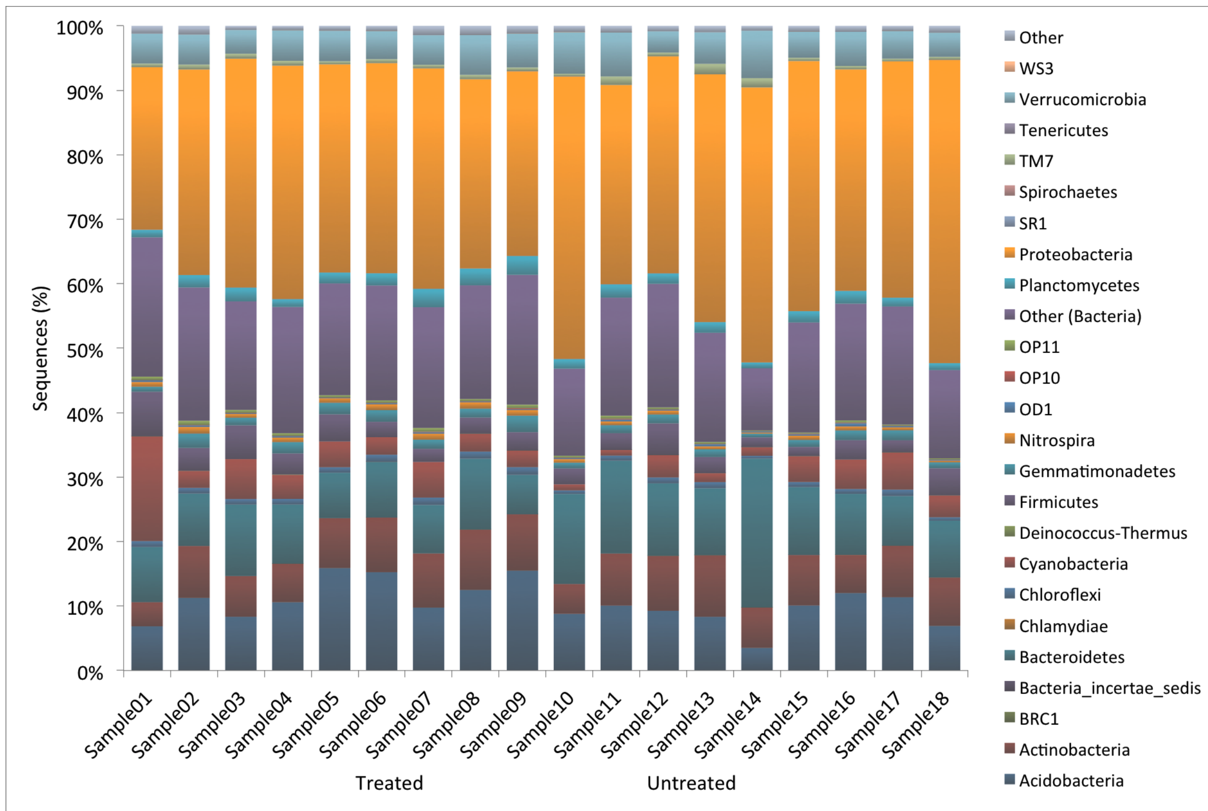
440 Figure 1



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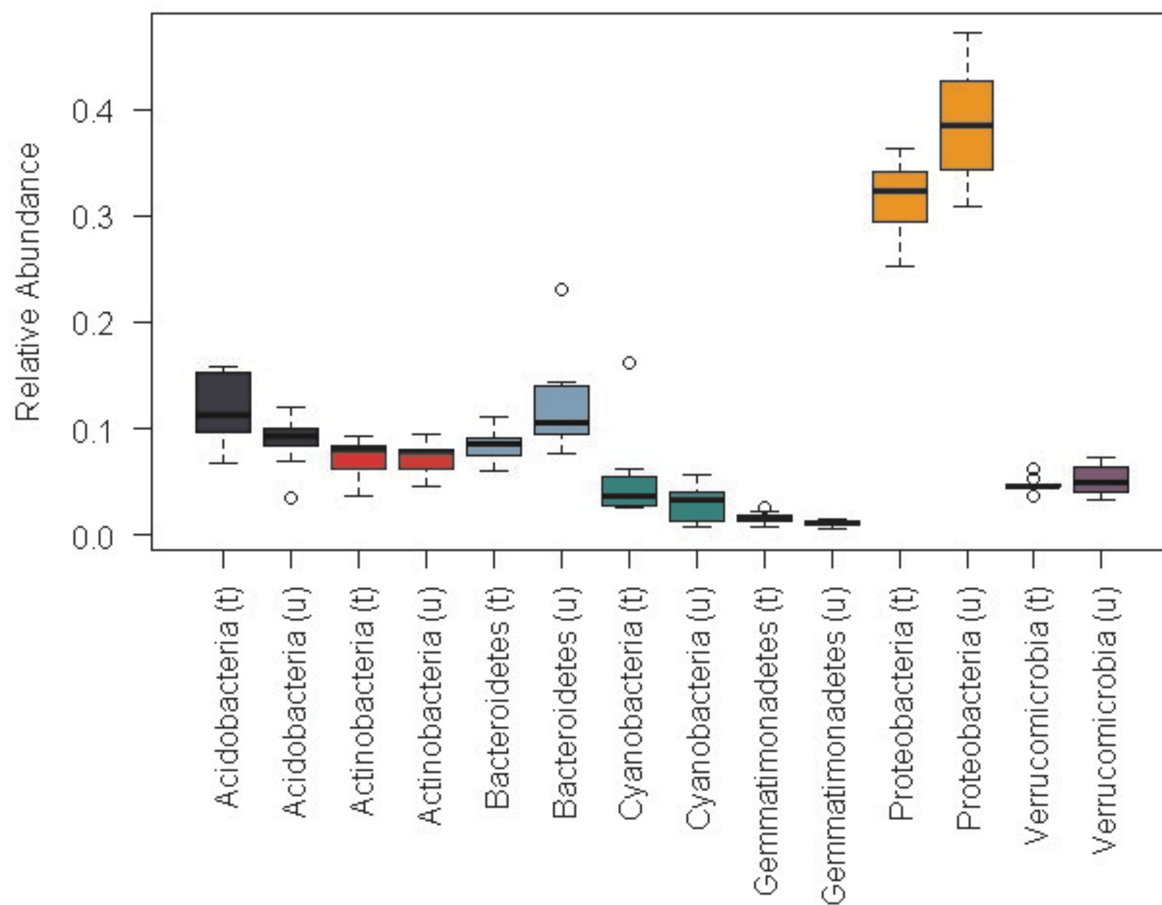
455 Figure 2



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458 Table 1

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Phylum	Genus*	Bonferroni Corrected *p*-value	OTU Abundance (Untreated)	OTU Abundance (Treated)
Proteobacteria	Duganella	4.41E-70	650	151
Proteobacteria	Dechloromonas	1.00E-21	109	6
Proteobacteria	Massilia	7.03E-15	113	16
Bacteroidetes	Flavobacterium	4.30E-12	131	29
Proteobacteria	Pseudomonas	5.64E-11	70	6
Proteobacteria	Methylibium	3.36E-09	95	18
Bacteroidetes	Flavobacterium	4.89E-09	43	0
Proteobacteria	Methylotenera	3.93E-08	125	36
Proteobacteria	Dechloromonas	2.12E-07	83	16
Cyanobacteria	(Cyanobacteria)	3.32E-07	216	383
Proteobacteria	(Burkholderiales)	4.05E-06	39	2
Proteobacteria	Massilia	1.95E-05	46	4
Proteobacteria	Pelomonas	6.47E-05	42	4
Proteobacteria	Skermanella	0.001764952	316	192
Proteobacteria	Rhizobium	0.007092573	23	0
Proteobacteria	Propionivibrio	0.007688197	24	1
Proteobacteria	Pseudorhodoferax	0.009619722	52	11
Proteobacteria	(Sphingomonadaceae)	0.015879182	152	75
Proteobacteria	Variovorax	0.032293728	52	13

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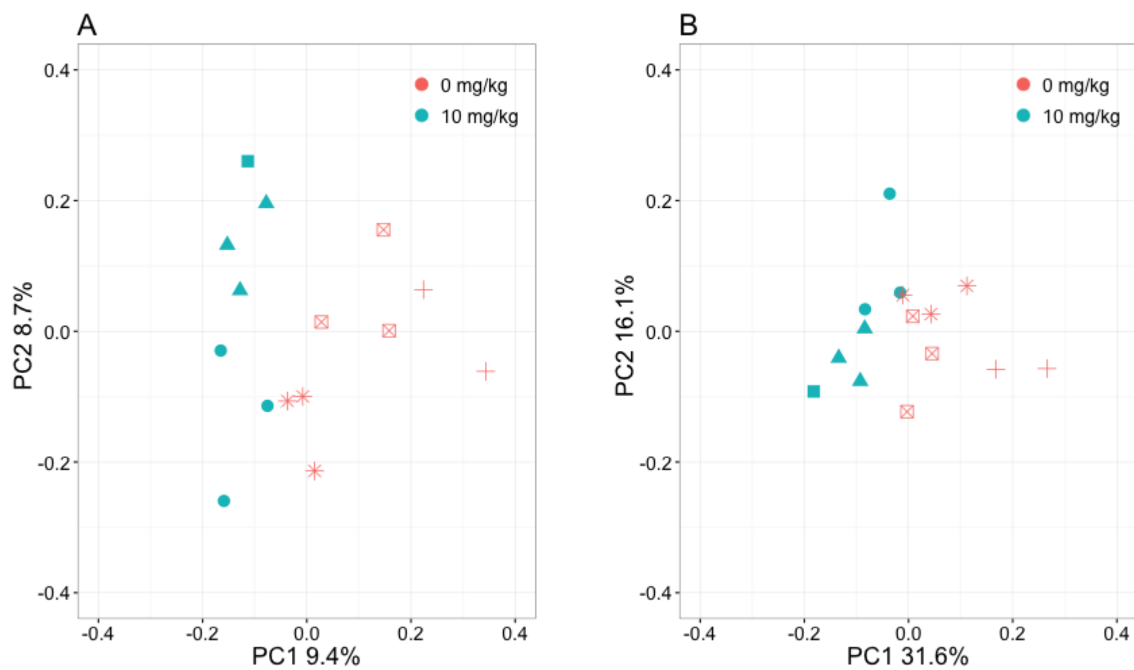
488 Figure 3



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492 Table S1. 16S rRNA OTU diversity and richness metrics for antibiotic treated and untreated
493 soil samples.

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	Observed Species Richness	Chaol	Phylogenetic Diversity (PD)	Shannon index (*H'*)
Average (Untreated)	5567.2	16417.9	620.7	11.2
Average (Treated)	5394.89	16279.9	600.2	11.2

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