FEMS Microbiology Ecology Advance Access published August 5, 2016

Title: Long-term antibiotic exposure in soil is associated with changes in microbial community structure and prevalence of class 1 integrons.

Authors: David W Cleary[a,b†], Alistair H Bishop[c], Lihong Zhang[d], Edward Topp[e], Elizabeth M H Wellington[f] and William H Gaze[d]

Affiliations:

a Academic Unit of Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, UK

b Institute for Life Sciences, Faculty of Natural and Environmental Sciences, University of Southampton, UK

c Defence Science Technology Laboratory, Porton Down, Salisbury UK

d European Centre for Environment and Human Health, University of Exeter Medical School, Knowledge Spa, Royal Cornwall Hospital, Truro, UK

e Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, Ontario, Canada

f School of Life Sciences, University of Warwick, Coventry, UK

[†]Corresponding author Tel: 0238120 8895, Fax: Email: d.w.cleary@soton.ac.uk

Running Title: Antibiotic Impacts in Soil

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

Introduction

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

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

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

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

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

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

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

Materials and Methods

Study Site and Sampling

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

DNA Extraction

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

Class 1 Integron Prevalence

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

16S rRNA Gene Amplification and 454 Sequencing

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

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

Quality Filtering, OTU Picking and Taxonomic Assignment

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

Alpha and Beta Diversity

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

Statistical Analysis

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

Results

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

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

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

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

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

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

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

Discussion

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

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

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

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

manure they concluded that observed differences were attributable to impacts of the antibiotic, and their results are therefore concordant with the findings of the present study. The low number of statistically significant OTUs reported here is a consequence of Bonferroni correction; by nature a conservative method of adjustment that has been highlighted previously to increase the likelihood of type II errors (false negatives) (Perneger, 1998). In fact, the separation observed between treatment groups in Figure 3 suggests that the limited number of statistically significant OTUs detailed in Table 1 represents only a fraction of those that have been impacted by the addition of antibiotics. A deeper level of sequencing would almost certainly resolve this and enable further identification of genera that are responsible for these changes. Only one OTU was shown to increase significantly, belonging to the Cyanobacteria (Table 1). This is an unexpected finding given the known sensitivity of members of this Class, which have seen their use in antibiotic environmental toxicity bioassays (Van der Grinten *et al*., 2010).

Rising antimicrobial resistance is clearly of global concern and a huge challenge for the medical and scientific community. Determing the impact of anthropogenic activities is a vital aspect of antibiotic stewardship in this regard. This study demonstrates, for the first time, that exposure of soil to veterinary antibiotics causes an increase in the prevalence of genetic elements, class 1 integrons, which can integrate a wide range of antibiotic resistance genes and are implicated in acquired resistance in clinically significant human pathogens. Many class 1 integrons also carry *sul1* in the integron backbone, which would select for the genetic element in the presence of sulfonaimides such as sulfamethazine. Additionally, a measurable shift in the structure of the microbial community was observed. Taxa decreasing in abundance are likely to be under strong selection for acquisition of resistance mechanisms, and class 1 integron carriage has been widely reported within the Proteobacteria; the phyla most affected in the present study. Further study is warranted to elucidate the link between

these two reported phenomena. Lastly it should be noted that the 10 mg kg^{-1} concentration used here is at the higher range of what would be expected in a realistic exposure scenario (Aust *et al.*, 2008; Heuer *et al.*, 2008). Therefore further experiments should be undertaken to verify that the present observations hold true under normal farming conditions.

Conflict of Interest

None to declare.

Funding

This work was financed by the UK Ministry of Defence (D.W.C.), Agriculture and Agri-Food Canada (E.T), Natural Environment Research Council (grant number NE/E004482/1 L.Z.) and the European Regional Development Fund (W.H.G.).

Acknowledgements

The authors wish to acknowledge Thomas J. Piggott for helpful discussions and for assistance with the production of figures.

References

Amos GCA, Zhang L, Hawkey PM, *et al*. (2014) Functional metagenomic analysis reveals rivers are a reservoir for diverse antibiotic resistance genes. *Vet Microbiol* **171**(3-4): 441-447. DOI:10.1016/j.vetmic.2014.02.017

Aust MO, Godlinski F, Travis GR, *et al*. (2008). Distribution of sulfamethazine, chlortetracycline and tylosin in manure and soil of Canadian feedlots after subtherapeutic use in cattle. *Environ Pollut* **156**:1243-1251.

Arias CA, Murray BE. (2009) Antibiotic-Resistant Bugs in the 21st Century: A Clinical Super-Challenge. *N Engl J Med* **360**:439-443. DOI: 10.1056/NEJMp0804651

Ashbolt NJ, Amézquita A, Backhaus T, *et al*. (2013) Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. *Environ Health Perspect* **121**:9 DOI:10.1289/ehp.1206316.

Baker GC, Smith JJ, Cowan DA. (2003) Review and re-analysis of domain-specific 16S primers. *J Microbiol Methods* **55**(3): 541-55. DOI:10.1016/j.mimet.2003.08.009

Byrne-Bailey KG, Gaze WH, Kay P, *et al*. (2009) Prevalence of sulfonamide resistance genes in bacterial isolates from manured agricultural soils and pig slurry in the United Kingdom. *Antimicrob Agents Chemother* **53**(2): 696-702. DOI: 10.1128/AAC.00652-07

Byrne-Bailey KG, Gaze WH, Zhang L, *et al*. (2011) Integron prevalence and diversity in manured soil. *Appl Environ Microb* **77**(2): 684-687. DOI: 10.1128/AEM.01425-10

Caporaso GJ, Kuczynski J, Stombaugh J, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* **7**: 335–336. DOI:10.1038/nmeth.f.303

Ding GC, Radl V, Schloter-Hai B, et al. (2014) Dynamics of Soil Bacterial Communities in Response to Repeated Application of Manure Containing Sulfadiazine. *PLoS ONE* **9**(3): e92958. doi: 10.1371/journal.pone.0092958.

Edgar RC. (2010) Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **26**(19): 2460-2461. DOI: 10.1093/bioinformatics/btq461

Finley RL, Collignon P, Larsson JDG, *et al*. (2013) The scourge of antibiotic resistance: the important role of the environment. *Clin Infect Dis* **57**(5): 704-710 DOI: 10.1093/cid/cit355

Gaze WH, Zhang L, Abdouslam NA, *et al*. (2011) Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME J* **5**(8): 1253-61. DOI: 10.1038/ismej.2011.15.

Gaze WH, Krone S, Larsson DGJ, *et al*. (2013) Influence of humans on the evolution and mobilization of the environmental resistome. *Emerg Infect Dis* **19**:7 DOI: 10.3201/eid1907.120871.

Gillings M, Gaze WH, Pruden A, *et al*. (2015) Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J* 9(6) 1269-79. doi: 10.1038/ismej.2014.226

Halling-Sørensen B, Jacobsen AM, Jensen J, *et al*. (2005) Dissipation and effects of chlortetracycline and tylosin in two agricultural soils: A field-scale study in southern Denmark. *Environ Toxicol Chem* **24**(4)**:**802-810. DOI: 10.1897/03-576.1

Hamscher G, Pawelzick HT, Höper H, Nau H. Antibiotics in Soil: Routes of Entry, Environmental Concentrations, Fate and Possible Effects. In: Kümmerer K (ed.). Pharmaceuticals in the Environment: Sources, Fate, Effects and Risks. Springer Science & Business Media, 2001, 139-148

Heuer H, Smalla K. (2007) Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. *Environ Microbiol* **9**(3):657-666. DOI: 10.1111/j.1462-2920.2006.01185.x

Heuer H, Focks A, Lamshöft M, *et al*. (2008) Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. *Soil Biol Biochem* **40**(7): 1892-1900. DOI:10.1016/j.soilbio.2008.03.014

Heuer H, Kopmann C, Binh CTT, *et al*. (2009) Spreading antibiotic resistance through spread manure: characteristics of a novel plasmid type with low %G+C content. *Environ Microbiol* **11**(4):937-949. DOI: 10.1111/j.1462-2920.2008.01819.x.

Heuer H, Schmitt H, Smalla K. (2011) Antibiotic resistance gene spread due to manure application on agricultural fields. *Curr Opin Microb* **14**(3): 236-243. DOI: 10.1016/j.mib.2011.04.009.

Hund-Rinke K, Simon M, Lukow T. (2004) Effects of tetracycline on the soil microflora: Function, diversity, resistance. *J Soils Sediments* **4**(1):11-16. DOI: 10.1007/BF02990823

Islas-Espinoza M, Reid BJ, Wexler M *et al*. (2012) Soil Bacterial Consortia and Previous Exposure Enhance the Biodegradation of Sulfonamides from Pig Manure. *Microb Ecol* **64**(1):140-151. DOI: 10.1007/s00248-012-0010-5.

Jechalke S, Focks A, Rosendahl I, *et al*. (2014) Structural and functional response of the soil bacterial community to application of manure from difloxacin-treated pigs. *FEMS Microbiol Ecol* **87**(1): 78-88. DOI: 10.1111/1574-6941.12191.

Jechalke S, Schreiter S, Wolters B *et al*. (2014) Widespread dissemination of class 1 integron components in soils and related ecosystems as revealed by cultivation-independent analysis. *Front. Microbiol*. **4**:420 doi:10.3389/fmicb.2013.00420

Kim M, Morrison M, Yu Z. (2011) Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. *J Microbiol Methods* **84**(1): 81-87. DOI: 10.1016/j.mimet.2010.10.020.

Knapp CW, Engemann CA, Hanson ML, *et al*. (2008) Indirect Evidence of Transposon-Mediated Selection of Antibiotic Resistance Genes in Aquatic Systems at Low-Level Oxytetracycline Exposures. *Environ Sci Technol* **42**(14): 5348-5353. DOI**:** 10.1021/es703199g

Kopmann C, Jechalke S, Rosendahl I, *et al*. (2013) Abundance and transferability of antibiotic resistance as related to the fate of sulfadiazine in maize rhizosphere and bulk soil. *FEMS Microbiol Ecol* **83**(1): 1125-134. DOI: 10.1111/j.1574-6941.2012.01458.x.

Kümmerer, K. (2004) Resistance in the environment. *J Antimicrob Chemother* **54**(2): 311-320. DOI: 10.1093/jac/dkh325

Manter DK, Weir TL, Vivanco JM. (2010) Negative Effects of Sample Pooling on PCR-Based Estimates of Soil Microbial Richness and Community Structure. *Appl Environ Microb* **76**(7): 2086-2090. DOI: 10.1128/AEM.03017-09

Partridge SR, Tsafnat G, Coiera E, Iredell JR. (2009) Gene cassettes and cassette arrays in mobile resistance integrons. *FEMS Microbiol Rev* **33**(4): 757-784. DOI: 10.1111/j.1574-6976.2009.00175.x

Perneger TV. (1998) What's wrong with Bonferroni adjustments. *BMJ* 316:1236 DOI: http://dx.doi.org/10.1136/bmj.316.7139.1236

Price MN, Dehal PS, Arkin AP. (2010) FastTree 2 approximately maximum-likelihood trees for large alignments. *PLoS ONE* **5**:3 e9490 DOI: 10.1371/journal.pone.0009490

Leonard AF, Zhang L, Balfour AJ, *et al*. (2015) Human recreational exposure to third-generation cephalosporin-resistant *Escherichia coli* in coastal bathing waters. *Environ Int* **82:** 92-100. doi:10.1016/j.envint.2015.02.013

Lozupone C, Knight R. (2005) UniFrac: a New Phylogenetic Method for Comparing Microbial Communities. *Appl Environ Microb* **71**(12): 8228-8235. DOI: 10.1128/AEM.71.12.8228-8235.2005

Reichel R, Rosendahl I, Peeters ETHM, *et al*. (2013) Effects of slurry from sulfadiazine- (SDZ) and difloxacin- (DIF) medicated pigs on the structural diversity of microorganisms in bulk and rhizosphere soil. *Soil Biol Biochem* **62**:82-91. DOI:10.1016/j.soilbio.2013.03.007

Shade A, Klimowicz AK, Spear RN, *et al*. (2013) Streptomycin application has no detectable effect on bacterial community structure in apple orchard soil. *Appl Environ Microb* **79**(21) : 6617-6625. DOI: 10.1128/AEM.02017-13

Shelver WL, Hakk H, Larsen GL, *et al*. (2010) Development of an ultra-high-pressure liquid chromatography tandem mass spectrometry multi-residue sulfonamide method and its application to water, manure slurry, and soils from swine rearing facilities. *Journal of Chromatogr A* **1217**(8): 1273-1282. DOI: 10.1016/j.chroma.2009.12.034.

Topp E, Chapman R, Devers-Lamrani M, *et al*. (2013) Accelerated biodegradation of veterinary antibiotics in agricultural soil following long-term exposure, and isolation of a sulfamethazine-degrading sp. *J Environ Qual* **42**(1): 173-178. DOI: 10.2134/jeq2012.0162.

van der Grinten E, Pikkemaat MG, van den Brandhof EJ, *et al*. (2010) Comparing the sensitivity of algal, cyanobacteria and bacterial bioassays to different groups of antibiotics. *Chemosphere* **80**(1): 1-6. DOI: 10.1016/j.chemosphere.2010.04.011

Wellington EMH, Boxall ABA, Cross P, *et al.* (2013) The role of the natural environment in the emergence of antibiotic resistance in Gram-negative bacteria *Lancet Infect Dis* **13**(2):155-165 DOI: 10.1016/S1473-3099(12)70317-1.

Westergaard K, Müller AK, Christensen S, *et al.* (2001) Effects of tylosin as a disturbace on the soil microbial community. *Soil Biol Biochem* **33**:15 2061-2071 DOI:10.1016/S0038-0717(01)00134-1

World Health Organization. (2014) Antimicrobial Resistance: Global Report on Surveillance World Health Organization, Geneva, Switzerland

Yergeau E, Bezemer TM, Hedlund K, *et al*. (2009) Influences of space, soil, nematodes and plants on microbial community composition of chalk grassland soils. *Environ Microbiol* **12**:8 2096–2106. DOI: 10.1111/j.1462-2920.2009.02053.x

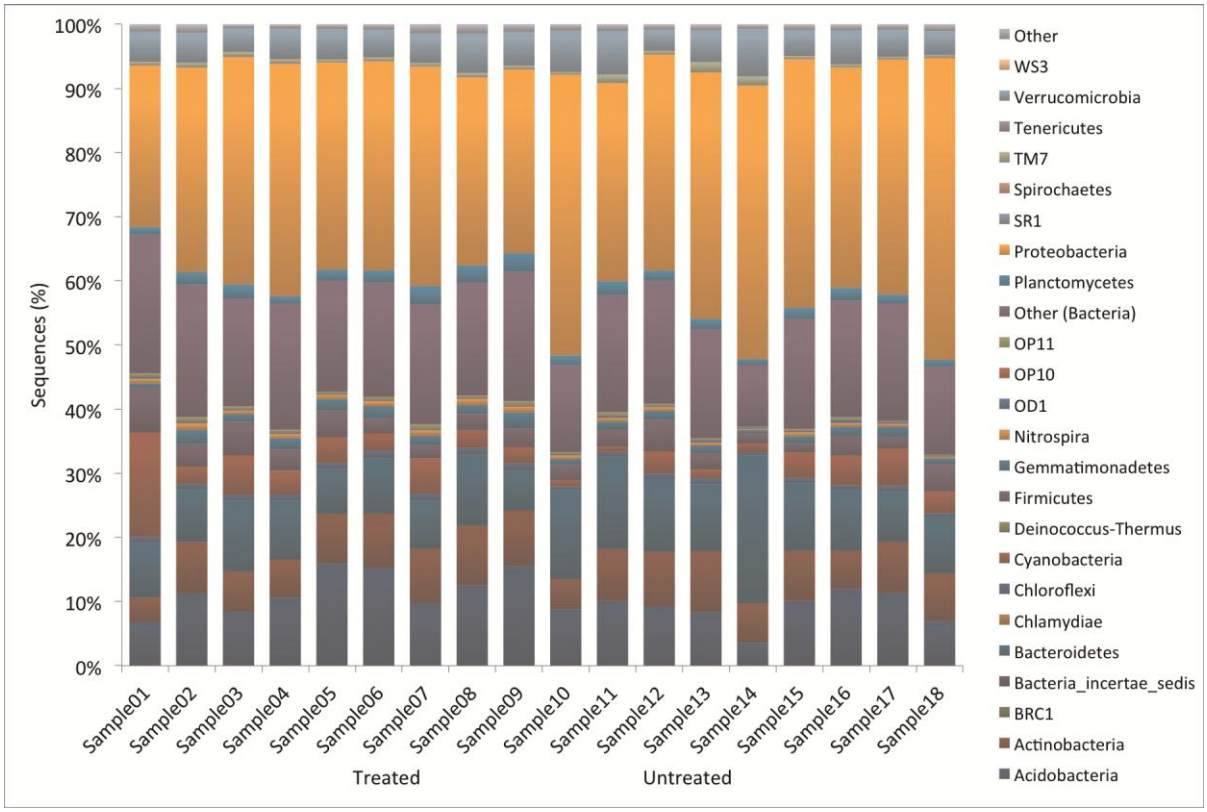

Figure 1. Relative abundance, expressed as a percentage, of major bacterial phyla in antibiotic treated (Samples 1 to 9) and untreated soil samples (10 to 18) using V1-3 of the 16S rRNA gene.

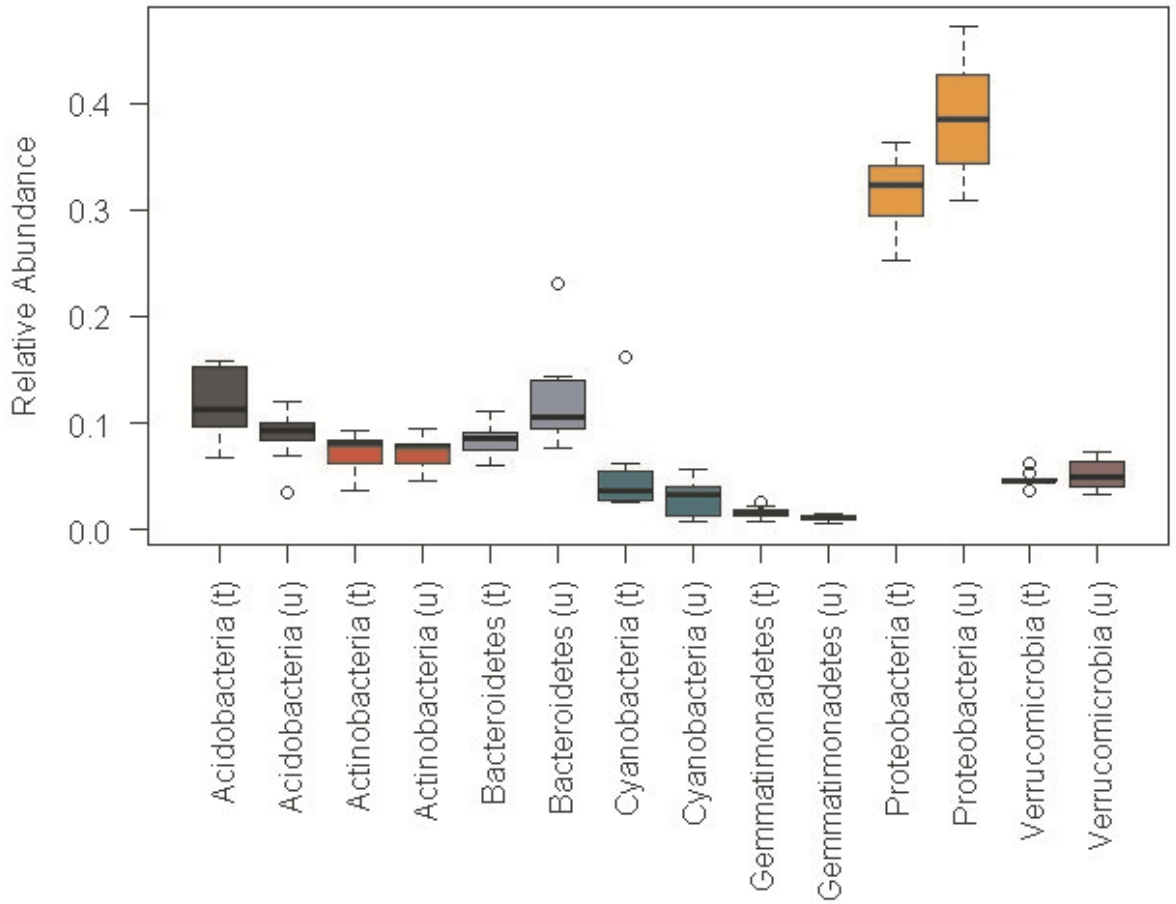

Figure 2. Box and whisker plots (n=9) comparing the relative abundances of each of the dominant phyla between antibiotic treated and untreated plots. T refers to treated, u to untreated. Boxplots show the first and third quartile (bottom and top lines of the box), the median (middle line of the box)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

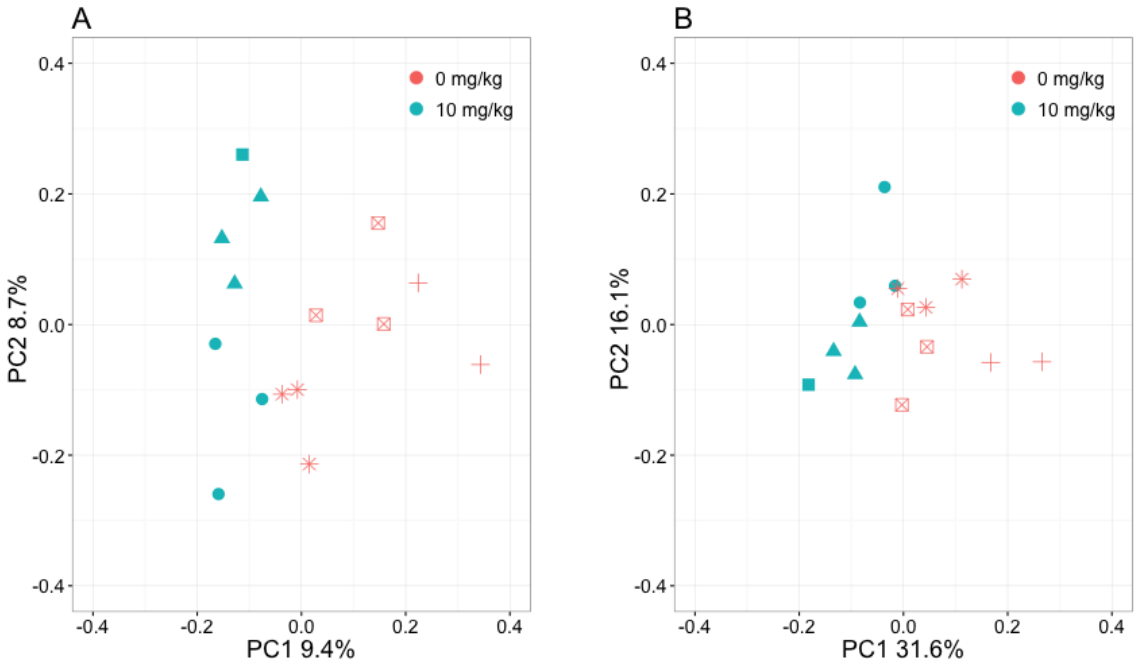

Figure 3. Principal component analysis showing OTU beta diversity of soils that have undergone antibiotic treatment (blue) compared to untreated (red) based on weighted (A) and unweighted UniFrac (B). Replicate samples of each plot are indicated by shape.

Table 1.

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