

University of Tennessee

Changes in Antibiotic Resistance of Soil Microbes During Human Decomposition

Victoria N. Beard

Undergraduate Thesis

Advisor: Dr. Jennifer M. DeBruyn, Graduate Student: Allison R. Mason

24 April 2020

Abstract

Since the introduction of commercial antibiotics, human consumption of these drugs has exponentially increased. This has led to an increase in antimicrobial resistant organisms, particularly those residing in the gastrointestinal tract. In nature, gastrointestinal-derived antibiotic residues and resistant microbes from the gastrointestinal tract can be transferred to the environment via fecal matter. Alternatively, this process may also occur during human decomposition. As a body decomposes, microbes and decomposition products are released into the surrounding soil. However, changes to the antibiotic resistance (ABR) of the soil community as a result of decomposition have not been extensively studied. Therefore, this study aimed to investigate changes in soil microbial ABR during human decomposition. Soil samples were taken from the soil surrounding thirteen donors at the Anthropology Research Facility prior to placement (T_0) and at the end of active decay (T_f). Soil microorganisms were then screened for functional resistance to five clinically relevant antibiotics via culturing on antibiotic-supplemented media, and specific resistance genes were tracked via qPCR. This study found significant changes in the abundance of colony forming units (CFUs) resistant to ciprofloxacin, streptomycin, and ampicillin in the soil community from T_0 to T_f . Additionally, it was noted that widespread resistance to tetracycline and sulfa drugs was present in soil communities prior to donor placement. Moreover, a variety of morphologies indicated that resistance was not confined to few species, but rather was found in a diverse array of taxa. Taken together, these results demonstrate that human decomposition increases antimicrobial resistance of soil microbial communities, at least temporarily, signaling that the soil microbiome is clinically relevant as a potential reservoir of antibiotic resistance.

Introduction

Antibiotic production and resistance have always been an important characteristic of environmental microbial communities. In complex microbial communities, microbes must compete for space and resources. As a consequence, some microbes produce antibiotics to overcome competition. Many of the same producers of these compounds also have mechanisms of resistance to ensure their survival (Clardy, Fischbach et al. 2009). When antibiotics are introduced to a microbial community, they create a selection pressure that favors the growth of resistant microorganisms (Thiele-Bruhn 2003, Sandegren 2014). The genes that are responsible for resistance are often mobile and may transfer to other microorganisms horizontally (Nwosu 2001, Agerso and Sandvang 2005). Thus, the development and dissemination of antibiotic resistance genes are a natural phenomenon in environments such as soil and water.

However, since the introduction of antibiotics into clinical practice, humans have exponentially increased their consumption of these drugs (Thiele-Bruhn 2003). Increased consumption of antibiotics has been positively correlated to the emergence and rise of resistant microbes in the human microbiome, particularly in the gastrointestinal tract (Thiele-Bruhn 2003, Ventola 2015). The gastrointestinal tract has the greatest microbial burden of any area in the body, and research has shown that microorganisms there interact with orally-administered drugs, often by chemically modifying or degrading these medications (Quigley 2013, Zimmermann, Zimmermann-Kogadeeva et al. 2019).

The antibiotic residues and resistant microbes in the gastrointestinal tract can then be transferred to the environment via fecal matter and decomposition (Keenan, Emmons et al. 2018, Perez-Valera, Kyselkova et al. 2019). The transfer of antibiotic residues from fecal matter to soil

and water environments has been studied extensively. The application of manure onto soil has been found to increase the number of microbes resistant to a variety of antibiotics, including sulfa drugs, amoxicillin, β -lactams and tetracycline; additionally, manure that has been supplemented with antibiotics leads to long-term increases in resistance of the soil community (Heuer, Krogerrecklenfort et al. 2002, Binh, Heuer et al. 2007, Udikovic-Kolic, Wichmann et al. 2014, Perez-Valera, Kyselkova et al. 2019). Moreover, as tissues breakdown and fluids are released during human decomposition, microbes from the cadaver enter the soil. This release of microbes into the local environment can greatly affect the microbial community (Carter, Yellowlees et al. 2007). This is evidenced by studies that show that human graves often have elevated number of microbes in surface soils, and these microbial communities can significantly differ from their composition prior to cadaver placement (Keenan, Emmons et al. 2018).

As a result, human decomposition creates a selection pressure within the soil microbial communities to develop mechanisms of resistance or to obtain them from the resistant microbes via horizontal gene transfer. In fact, horizontal gene transfer has been categorized as a major facilitator of the spread of antibiotic resistance genes (ARGs) (Nwosu 2001, Agerso and Sandvang 2005). Thus, the soil microbial communities may become reservoirs of antibiotic resistance as a result of human decomposition. Within this reservoir, new pathogens may arise and may be transferred to other microbial communities like the human microbiome via water contamination. Consequently, the antibiotic resistance of the environment is clinically relevant as a potential source of emergent pathogens (D'Costa, McGrann et al. 2006). This emergence of antibiotic resistance in microorganisms poses a major threat to global health outcomes (CDC 2011). When resistance increases, it results in infections that are difficult to treat and more incidences of infection-related death (Thiele-Bruhn 2003).

However, the direct impacts of decomposition on the resistance of soil microorganisms is unclear. Therefore, the aim of this study is to investigate short-term changes in the antibiotic resistance of the soil microbial community during active decomposition of human donors. We hypothesize that human decomposition will increase the resistance of the soil community. To investigate this hypothesis, human donors were placed at the Anthropology Research Facility. Soil microbes were screened for resistance to ampicillin, streptomycin, ciprofloxacin, tetracycline and sulfamethazine via culturing on plates supplemented with the aforementioned antibiotics. Additionally, qPCR was used to quantify changes in abundance of specific genes encoding antibiotic resistance.

Methods and Materials

Field Location and Donor Placement

Soil samples were collected as part of an ongoing research project at the Anthropology Research Facility (ARF) at the University of Tennessee. This facility is located in Knoxville, Tennessee (35°56'28" N, 83°56'25" W) and comprises an outdoor research laboratory of approximately 2.5 acres. It is a deciduous forest with a soil type of finely textured clay. The annual mean high temperature is 20.7°C, and the annual mean low temperature is 6.4°C. The mean relative humidity is 72%, while the mean yearly rainfall is 131.9 cm. These climate data were collected from the Alcoa Highway Weather Station, approximately 4.8 km from the ARF.

This study utilized thirteen individuals, which were donated to the University of Tennessee Forensic Anthropology Center for the W.M. Bass Donated Skeletal Collection. Given that no living human subjects were used, this project was exempt from review by the University of Tennessee Institutional Review Board. The University of Tennessee protocol for accepting donors ensured that these individuals were not carriers of communicable diseases. No preference

for sex, age, weight, ethnicity or medical history was employed. All donors utilized were Caucasian. The donors were not autopsied or embalmed, and they had no significant bodily trauma. They were refrigerated upon arrival and were placed in the ARF within three days of arrival. The donors in this study were placed prone on soil, without clothing or other covering. Placement dates range from February to September of 2019 (**Table 1**).

Table 1. Donor and placement details. The date of placement corresponds with the date of initial sampling. The date of final sampling occurred at the end of active decay, as defined by the end of fluid release.

Donor	Date of Placement	Date of Final Sampling	Medical History
TOX001	25 Feb. 2019	29 May 2019	Respiratory diseases, gastrointestinal diseases, cardiovascular diseases, metabolic diseases, cancer, tobacco abuse
TOX002	13 Mar. 2019	13 Apr. 2019	Metabolic diseases, cancer, tobacco and alcohol abuse
TOX003	2 Apr. 2019	29 Apr. 2019	Respiratory diseases, pancreatic diseases, tobacco and alcohol abuse
TOX004	23 Apr. 2019	8 May 2019	Cancer, tobacco abuse
TOX005	26 Apr. 2019	28 May 2019	Respiratory diseases, cancer
TOX006	13 May 2019	28 May 2019	Respiratory diseases, cardiovascular diseases, cancer, renal disease, tobacco abuse
TOX007	16 May 2019	3 Jun. 2019	Cardiovascular diseases, tobacco abuse
TOX008	10 Jun. 2019	1 Jul. 2019	Gastrointestinal diseases, cardiovascular diseases, cancer
TOX009	24 Jun. 2019	7 Jul. 2019	Cancer, tobacco abuse
TOX010	9 Jul. 2019	28 Aug. 2019	Respiratory diseases, metabolic diseases
TOX011	31 Jul. 2019	19 Aug. 2019	Cancer, cardiovascular diseases, motor diseases
TOX012	20 Sept. 2019	7 Oct. 2017	Metabolic diseases, renal disease, cardiovascular diseases
TOX013	20 Sept. 2019	15 Oct. 2019	Sleep disorders, chronic migraines

Soil Sampling

Soil samples were collected immediately prior to donor placement (defined as T_0), as well as at the end stage of active decomposition (defined at T_F). The end of active decomposition was determined by a cessation of decomposition fluid release from the abdomen into the

surrounding soil and significant mass loss from the donor. The soil samples were taken using modified disposable syringes, which were made by removing the end of a 10mL sterile syringe with a sterile scalpel to create single-use probes. For each sample, 5 to 8 5-centimeter soil cores were collected. All grave samples were taken within the area of human decomposition-impacted soils, termed the cadaver decomposition island (CDI). Cores were then homogenized, and debris was removed before being flash-frozen in liquid nitrogen and stored at -80°C until further analyses.

The gravimetric moisture of each sample was determined by weighing two to three grams of wet soil in an aluminum tin and drying it in an oven at 105°C for three days. It was then re-weighed to find the dry weight of the soil, and gravimetric moisture content was calculated using the following equation:

$$\theta g = \frac{\text{Weight of Wet Soil} - \text{Weight of Dry Soil}}{\text{Weight of Dry Soil}}$$

The gravimetric moisture content of the soil was used to determine the dry weight of soil for samples used in culturing and qPCR experiments by re-arranging the above equation into:

$$\text{Weight of Dry Soil} = \frac{\text{Weight of Wet Soil}}{\theta g + 1}$$

Culturing

Agar plates for antibiotic resistance screening were prepared using the following antibiotics: ampicillin, streptomycin, tetracycline, ciprofloxacin, and sulfamethazine. Stock solutions of each antibiotic were prepared in water and filter sterilized. These stock solutions were then added to respective aliquots of sterile Luria-Bertani (LB) media to bring the final concentrations described in **Table 2**. These concentrations were chosen based on previous

studies assessing antibiotic resistance (Esiobu, Armenta et al. 2002, Sandegren 2014).

Additionally, LB plates without antibiotic were used as a control.

Table 2. *Antibiotic Information. This table contains the details of each antibiotic used in resistance screening.*

Antibiotic Name	Manufacturer	Purity/Grade	Stock Solution	Media Concentration
Ampicillin	VWR Life Sci	USP	50 mg mL ⁻¹	150 µg mL ⁻¹
Ciprofloxacin	Acros Org	≥98%	10 mg mL ⁻¹	10 µg mL ⁻¹
Streptomycin	VWR Life Sci	USP	10 mg mL ⁻¹	50 µg mL ⁻¹
Sulfamethazine	MP Biomed	≥90%	60 mg mL ⁻¹	60 µg mL ⁻¹
Tetracycline	Millipore Sigma	USP	10 mg mL ⁻¹	10 µg mL ⁻¹

Soil solutions were prepared using sterile 1X phosphate-buffered saline (PBS) at a pH of 7.4. One gram of frozen soil was weighed and added to 10 mL PBS in a sterile 15 mL falcon tube. Tubes were then vortexed for 10 seconds to suspend microorganisms in the solution. The soil solution was rested for five minutes to allow larger soil particles to settle. Each solution was then serially diluted in PBS to concentrations of 10⁻⁴, 10⁻⁵, and 10⁻⁶. 100 µL of each dilution was spread, using aseptic technique, on each of the six media types in triplicate to give final dilutions of 10⁻⁵, 10⁻⁶, and 10⁻⁷. Plates were wrapped in Parafilm® and incubated in a dark incubator at 25°C for three days. This temperature was chosen as soil microbes grow well at or near 25°C (Janssen 2008). Then, colonies were counted. Overgrown plates, defined as having more than 300 CFUs, were given the distinction “U,” while for all others, the numerical value of the colony count was recorded.

Statistical Analysis

To allow comparison between different soil samples, colony forming units (CFU) were normalized per gram of dry soil. The acceptable colony count values for this calculation were 25 to 300 colonies per plate. The CFU values of T₀ and T_F for each donor and media type were compared using paired T-tests.

qPCR

DNA was extracted from flash-frozen soil samples using the DNeasy® PowerLyzer® PowerSoil® Kit following manufacturer instructions for high organic soils and extracts were stored at -20°C prior to analysis. Six genes that confer antibiotic resistance to ampicillin, streptomycin, and ciprofloxacin were selected for analysis via qPCR: *bla_{TEM}*, *mecA*, *qnrA*, *qnrB*, *strA*, *strB*. Primers sequences were selected from previous studies and were ordered from Invitrogen (**Table 3**).

Table 3. Primers. This table lists all primers used for PCR and qPCR in this study. *T_m* is the annealing temperature for each primer.

Gene	Directionality	Sequence	T _m (°C)	Reference
<i>bla_{TEM}</i>	Forward	ATAAAATTCTTGAAGACGAAA	53	(Bertrand, Weill et al. 2006)
	Reverse	GACAGTTACCAATGCTTAATC		
<i>mecA</i>	Forward	AGTTAGATTGGGATCATAGCGTC	53	(Bertrand, Weill et al. 2006)
	Reverse	TTGTCTGCCAGTTTCTCCTTG		
<i>qnrA</i>	Forward	ATTTCTCACGCCAGGATTTG	53	(Liu, Yang et al. 2013)
	Reverse	GATCGGCAAAGGTTAGGTCA		
<i>qnrB</i>	Forward	GATCGTGAAAGCCAGAAAGG	50	(Liu, Yang et al. 2013)
	Reverse	ATGAGCAACGATGCCTGGTA		
<i>strA</i>	Forward	CTTGGTGATAACGGCAATTC	55	(Srinivasan, Nam et al. 2008)
	Reverse	CCAATCGCAGATAGAAGGC		
<i>strB</i>	Forward	ATCGTCAAGGGATTGAAACC	56	(Srinivasan, Nam et al. 2008)
	Reverse	GGATCGTAGAACATATTGGC		

To confirm the presence of these genes, samples containing high levels of resistant microbes were selected and PCR was performed using Platinum™ Superfi™ PCR Master Mix. These PCR products were run via electrophoresis on a 1.7% agarose gel and visualized under a UV light (**Figure 5**). After confirmation that these genes were present in the samples, another

PCR was performed with Platinum™ *Taq* DNA Polymerase High Fidelity to produce gene products with 3' adenine overhangs.

Products for the gene *qnrB* were purified using the QIAquick® PCR Purification Kit. This gene was then ligated into plasmids and used to transform One Shot® Top 10 Chemically Competent *E. coli* with the TOPO TA Cloning® Kit for Sequencing. The transformed *E. coli* were grown in an overnight LB liquid media culture at 37°C and shaking at 200 rpm. Transformants were then plated on LB with 100 µg mL⁻¹ ampicillin and grown at 25°C for 72 hours to identify organisms with the desired plasmid. Five colonies from each plate were selected for sequencing. They were inoculated into LB liquid culture and grown overnight at 37°C and shaking at 200 rpm.

Plasmids were extracted from the *E. coli* with the PureLink™ Quick Plasmid MiniPrep Kit and submitted to Eurofins Genomics for sequencing. Plasmid sequences were aligned with a known *Klebsiella pneumoniae qnrB* sequence obtained from NCBI [Gene ID: 15486659] using the Clustal Omega Multiple Sequence Alignment tool. To confirm alignment results, PCR was performed on the plasmids with Platinum™ *Taq* DNA Polymerase High Fidelity and run through a 1.7% agarose gel. Bands were visualized under a UV light. Extracted plasmids were quantified with the Qubit™ dsDNA HS Assay Kit from Invitrogen and a Qubit® 3.0 Fluorometer. The concentration of the sample was found using this equation, where QF is the reading obtained from the Qubit® 3.0 Fluorometer:

$$\text{Concentration of sample} = QF \times \frac{\text{Total volume of sample}}{\text{Volume of sample used}}$$

The plasmid copy number was determined using the Thermo Fisher Scientific DNA Copy Number and Dilution Calculator. The vector size was 3956 base pairs, and the *qnrB* gene was estimated to be 550 base pairs. The total plasmid size was therefore 4506 base pairs.

qPCR was then performed using the Maxima SYBR Green qPCR Master Mix (2X) and the Bio-Rad CFX96 Detection System. Gene copy abundances were normalized to the dry weight of soil from which DNA was extracted. Then, *qnrB* abundances at the initial and final timepoints were compared using T-tests.

Results

Colony-forming units (CFUs) per gram dry soil were calculated for all samples. After T-tests were performed, thirty-nine of seventy-eight samples had significant differences between the initial and final time points. Human decomposition resulted a significant increase ciprofloxacin resistant CFUs in ten of thirteen donors' soils (**Table 7**). There was a significant increase in CFUs resistant to ampicillin and streptomycin conditions for nine and eight donors' soil samples, respectively (**Tables 4, 5**). The CFUs resistant to tetracycline and sulfamethazine had the fewest of significant differences between the initial and final sampling, with each having four instances of a significant increase (**Tables 6, 8**). However, human decomposition significantly increased overall CFU abundances in five of thirteen samples (**Table 9**). When eliminating donors with significant increases in the control condition, the number of significant differences between T_0 and T_F for the other conditions are as follows: seven donors had significant differences in ampicillin resistance, six had significant differences in streptomycin resistance, six had significant differences in ciprofloxacin resistance, and two had significant differences in tetracycline and sulfamethazine resistance. Overall, the soil samples from donors TOX010, TOX012, and TOX013 had the greatest number of significant increases in resistant

CFUs. Meanwhile, soil samples from donors TOX007 and TOX009 had the fewest instances of a significant increase in resistant CFUs.

Table 4. *T*-test results for ampicillin-resistant colony forming units at initial placement (T_0) versus the end of active decomposition (T_F). M_0 indicates the mean number of CFUs per gram dry weight of soil at T_0 . M_F indicates the mean number of CFUs per gram dry weight of soil at T_F . Significant *p*-values ($p \leq 0.05$) are bolded.

Donor	M_0	M_F	T-value	P-value
TOX001	0	38767364.15	2.776	0.0536
TOX002	0	21814743.03	2.776	0.0208
TOX003	0	12084264.61	12.71	0.0484
TOX004	0	461632555.6	4.303	0.0130
TOX005	0	39106009.4	2.570	0.0032
TOX006	0	3742057896	4.303	0.0007
TOX007	21578001.82	28495123.2	12.71	0.8860
TOX008	0	30823389.57	2.776	0.0711
TOX009	0	0	—	—
TOX010	3434474.667	26478913.14	4.303	0.0470
TOX011	0	73305963.17	4.303	0.0074
TOX012	0	23643641.75	4.303	0.0058
TOX013	0	33212522.57	4.303	0.0373

Table 5. *T*-test results for streptomycin-resistant colony forming units at initial placement (T_0) versus the end of active decomposition (T_F). M_0 indicates the mean number of CFUs per gram dry weight of soil at T_0 . M_F indicates the mean number of CFUs per gram dry weight of soil at T_F . Significant *p*-values ($p \leq 0.05$) are bolded.

Donor	M_0	M_F	T-value	P-value
TOX001	3757277.622	23804521.85	4.303	0.0380
TOX002	5389514.423	51864370.37	3.182	0.0333
TOX003	0	12885011.85	4.303	0.0020
TOX004	0	100980168	4.303	0.0107
TOX005	0	37403943.18	3.182	0.0450
TOX006	29596070.05	375084206.9	4.303	0.1240
TOX007	3490559.117	9427249.745	3.182	0.2902
TOX008	0	56743311.06	2.571	0.0639
TOX009	0	0	—	—
TOX010	2136075.708	60799121.29	3.182	0.0394
TOX011	0	48013262.43	4.303	0.0711
TOX012	0	11561045.41	4.303	0.0432
TOX013	0	4210038.072	4.303	0.0004

Table 6. *T*-test results for tetracycline-resistant colony forming units at initial placement (T_0) versus the end of active decomposition (T_F). M_0 indicates the mean number of CFUs per gram dry weight of soil at T_0 . M_F indicates the mean number of CFUs per gram dry weight of soil at T_F . Significant *p*-values ($p \leq 0.05$) are bolded.

Donor	M_0	M_F	T-value	P-value
TOX001	0	39204590.06	3.182	0.1311
TOX002	0	36116324.29	2.571	0.0286
TOX003	0	16272308.78	3.182	0.1161
TOX004	41797607.38	813325034.9	2.776	0.0927
TOX005	51443758.2	297515641.3	4.303	0.4771
TOX006	326629910.5	500697887.5	4.303	0.3582

TOX007	118361686.4	152310216	2.571	0.6731
TOX008	57139857.44	1636177651	4.303	0.3789
TOX009	1697799779	131732719.2	2.776	0.1572
TOX010	25591024.65	1156416505	2.571	0.0258
TOX011	20512264.37	1429680627	2.571	0.0449
TOX012	0	24969250.34	12.71	0.1579
TOX013	0	21143746.76	4.303	0.0409

Table 7. *T*-test results for ciprofloxacin-resistant colony forming units at initial placement (T_0) versus the end of active decomposition (T_F). M_0 indicates the mean number of CFUs per gram dry weight of soil at T_0 . M_F indicates the mean number of CFUs per gram dry weight of soil at T_F . Significant *p*-values ($p \leq 0.05$) are bolded.

Donor	M_0	M_F	T-value	P-value
TOX001	0	29643916.8	2.776	0.0329
TOX002	0	3804897.04	4.303	0.0052
TOX003	0	5667634.08	4.303	0.0511
TOX004	0	84261597.11	4.303	0.0572
TOX005	0	6033340.446	4.303	0.0440
TOX006	0	75983100.47	4.303	0.0219
TOX007	0	27932591.84	4.303	0.0062
TOX008	0	30990363.51	2.776	0.0050
TOX009	0	0	—	—
TOX010	0	3023789.26	4.303	0.0004
TOX011	0	126034813.9	4.303	0.0088
TOX012	0	6867087.126	4.303	0.0235
TOX013	0	16138479.28	4.303	0.0032

Table 8. *T*-test results for sulfamethazine-resistant colony forming units at initial placement (T_0) versus the end of active decomposition (T_F). M_0 indicates the mean number of CFUs per gram dry weight of soil at T_0 . M_F indicates the mean number of CFUs per gram dry weight of soil at T_F . Significant *p*-values ($p \leq 0.05$) are bolded.

Donor	M_0	M_F	T-value	P-value
TOX001	47779112.45	134286651.6	2.776	0.3170
TOX002	4422165.68	60183807.95	12.71	0.5966
TOX003	3849282.158	23157598.62	4.303	0.0805
TOX004	9.52389E+14	6.15243E+14	4.303	0.0607
TOX005	9.0541E+14	1.01294E+15	12.71	0.3171
TOX006	7.39902E+16	1.22978E+17	4.303	0.0190
TOX007	1.57868E+17	2.26952E+17	12.71	0.1653
TOX008	1.97106E+17	2.02038E+17	4.303	0.9643
TOX009	1.45744E+17	3.80301E+17	4.303	0.0248
TOX010	1.74656E+17	2.11273E+17	4.303	0.4444
TOX011	3.92827E+15	8.31658E+14	4.303	0.0002
TOX012	239116733.3	399855706.1	4.303	0.0059
TOX013	408031111.2	612794430.5	4.303	0.1148

Table 9. *T*-test results for colony forming units at initial placement (T_0) versus the end of active decomposition (T_F) under control conditions. M_0 indicates the mean number of CFUs per gram dry weight of soil at T_0 . M_F indicates the mean number of CFUs per gram dry weight of soil at T_F . Significant *p*-values ($p \leq 0.05$) are bolded.

Donor	M_0	M_F	T-value	P-value
TOX001	40040242.13	116630011.9	3.182	0.0227
TOX002	14740552.27	176655934	4.303	0.0013
TOX003	92497675.74	96526892.92	4.303	0.6934

TOX004	4.49313E+14	6.92149E+14	12.71	0.5281
TOX005	9.98009E+14	4.89863E+14	12.71	0.2122
TOX006	7.79438E+16	1.43621E+17	4.303	0.2883
TOX007	1.50729E+17	2.19969E+17	4.303	0.0640
TOX008	8.49235E+16	2.538E+17	4.303	0.0492
TOX009	9.89863E+16	3.60823E+17	4.303	0.0204
TOX010	8.96314E+16	1.1727E+17	4.303	0.3431
TOX011	1.28803E+15	1.57758E+15	4.303	0.0021
TOX012	242685639.8	321623067.9	4.303	0.0847
TOX013	251376309.6	388259066.7	4.303	0.0984

Additionally, there were several antibiotics that the soils did not generally have microbial resistance for prior to donor placement. These include ampicillin, streptomycin and ciprofloxacin. In contrast, there was widespread microbial resistance prior to donor placement for both tetracycline and sulfamethazine. Overall, there was a general trend of increasing resistance from initial to final sampling for ampicillin, streptomycin, and ciprofloxacin. For tetracycline and sulfamethazine, the general trend was no significant change from initial to final sampling resistance (**Figure 1**). The values of the change between the resistant CFUs from the initial time point to the final time point, called delta, for each antibiotic are shown in **Figures 2 and 3**. The range of the delta values for each antibiotic varied considerably. Ampicillin, ciprofloxacin, and streptomycin plates had the least amount of variability in changes from the initial time point to the final time point. Tetracycline plates had a moderate amount of variability, while the sulfamethazine and control plates had the greatest amount of variability. Additionally, the ciprofloxacin plates had the lowest mean delta values, while the sulfamethazine and control plates had noticeably higher delta values than the other antibiotics. Plate culture colonies had a variety of morphologies that included circular, undulate, and filamentous forms, and some colonies also produced pigments such as red or yellow (**Figure 4**).

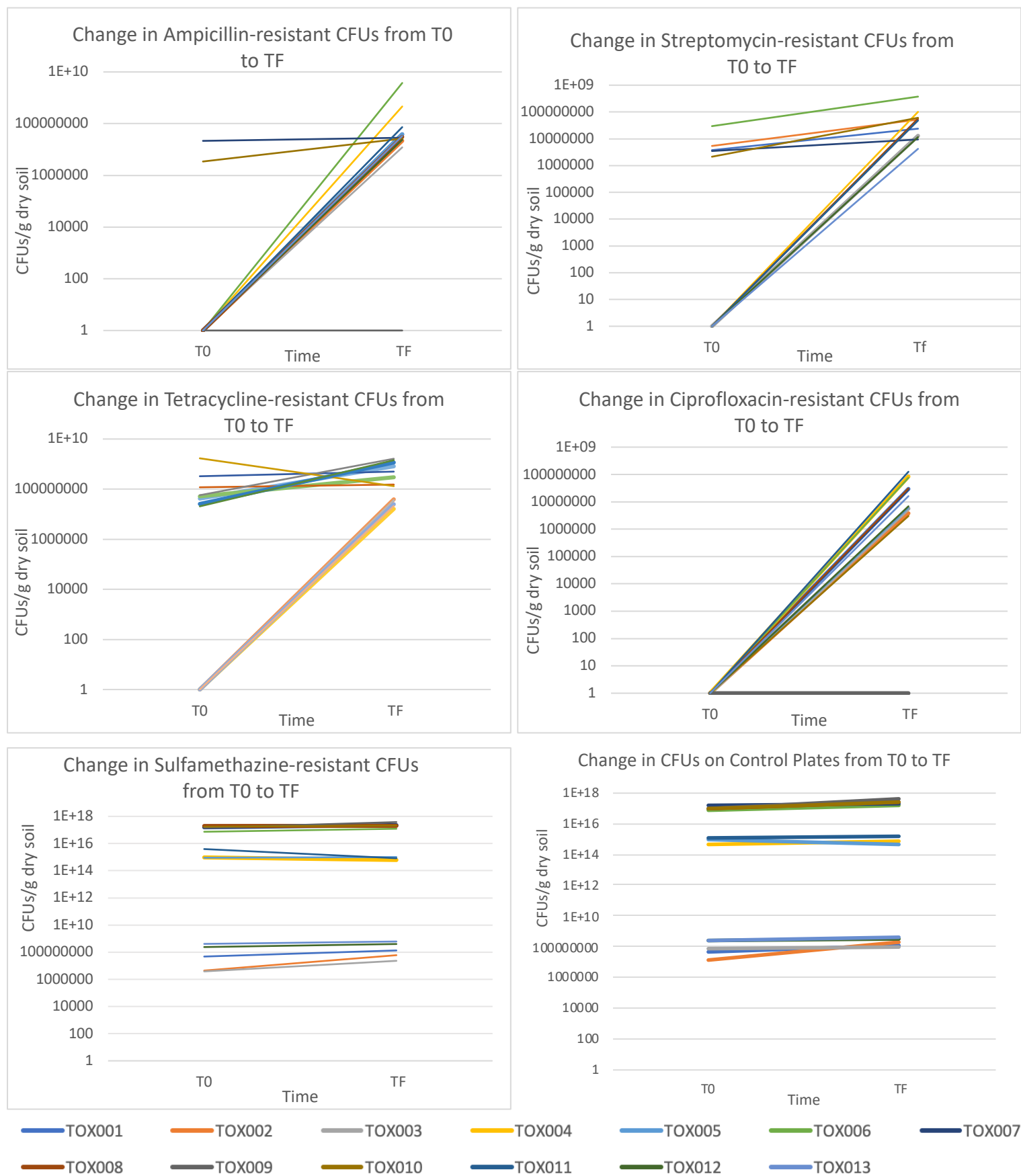


Figure 1. Changes in resistant CFUs from initial to final sampling for five antibiotics and a control condition.

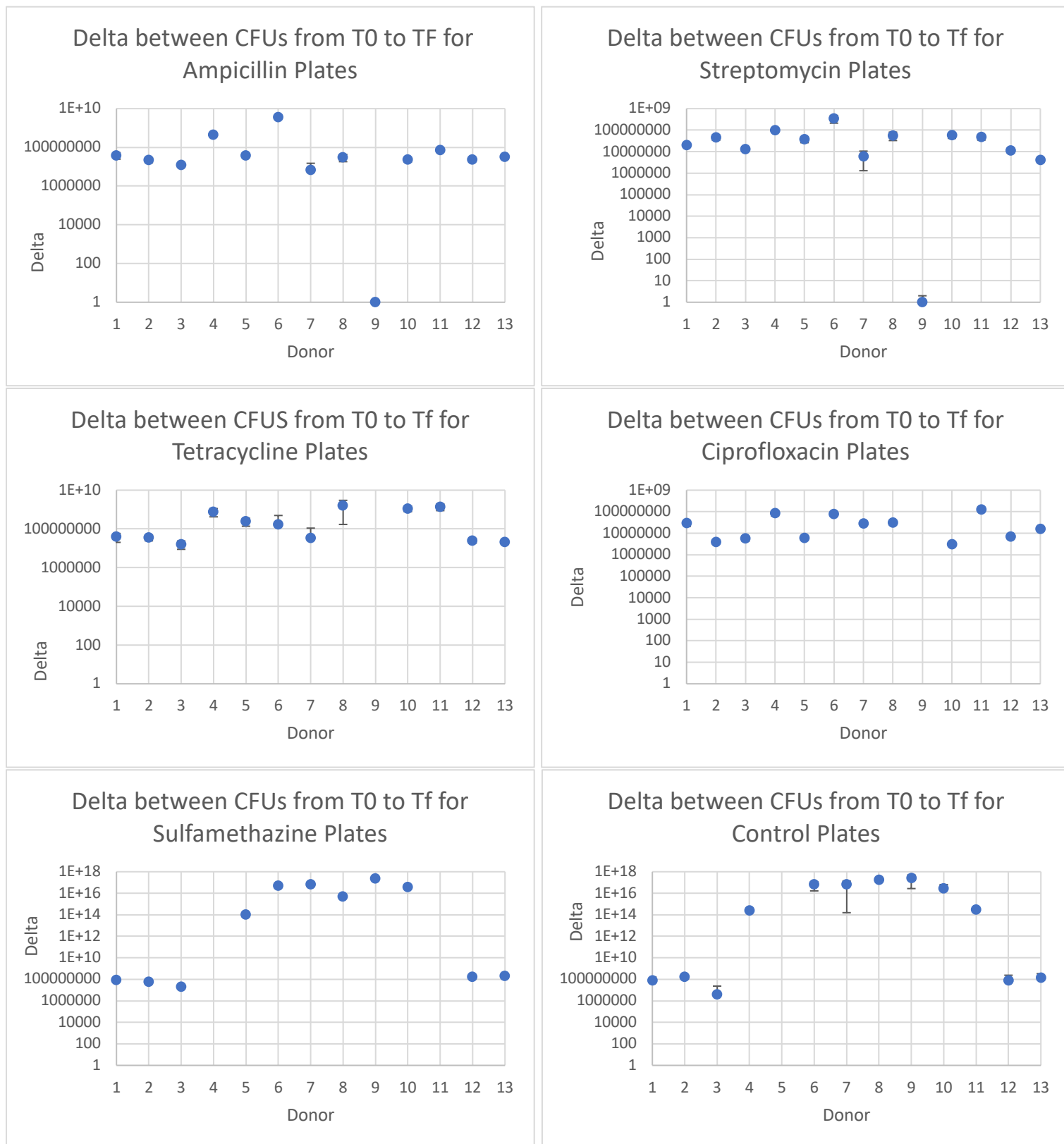


Figure 2. Mean change (delta) between the cultivable CFUs at the initial time point (T_0) and the final time point (T_F) for each donor.

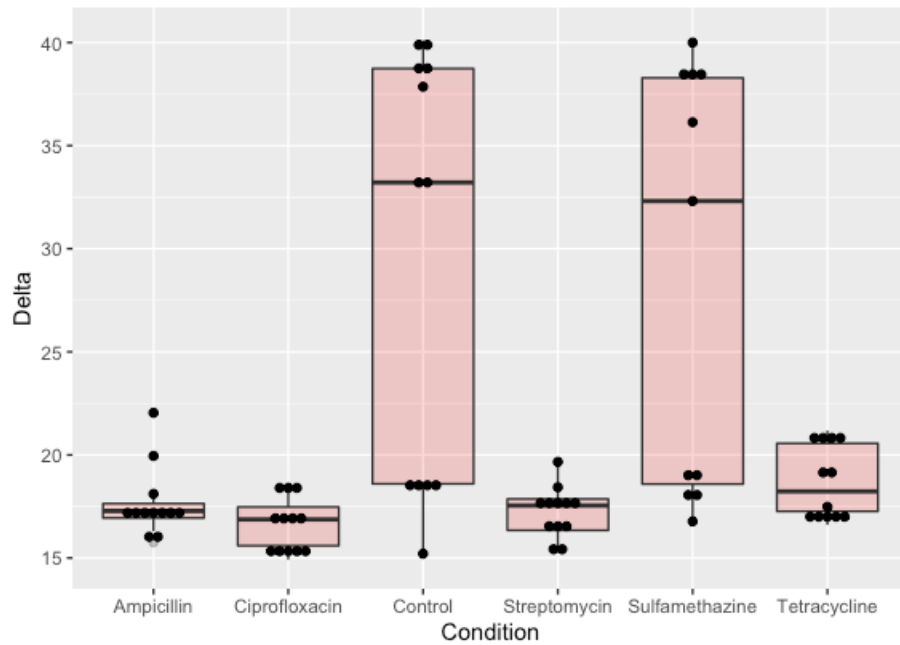


Figure 3. Deltas between the cultivable CFUs at the initial time point (T_0) and the final time point (T_F) for each donor. The y-axis is represented on a logarithmic scale.

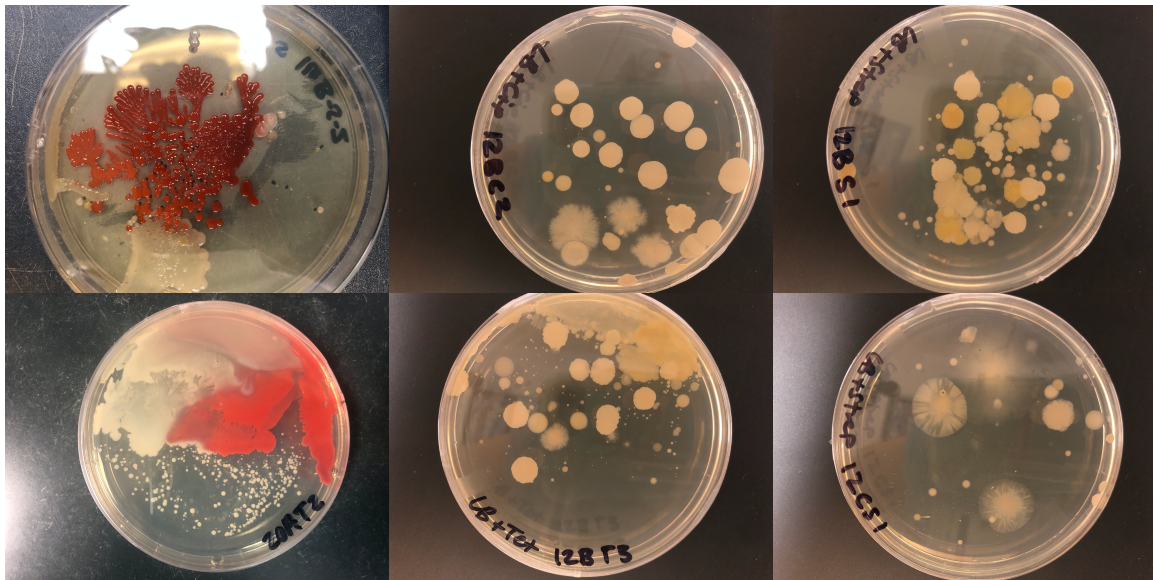


Figure 4. Six plates with colonies that display a variety of morphologies and pigmentation.

PCR was performed to investigate the presence of six antibiotic resistance genes among DNA samples with the highest number of resistant CFUs from the previous experiment (**Figure 5**). Primers for the gene *bla_{TEM}* in the first two sample lanes had unspecific amplification, resulting in numerous bands, as well as for the gene *mecA* in sample lanes three and four. Both of these genes confer ampicillin resistance. This PCR did confirm the existence of the genes *qnrA*, *qnrB*, *strA*, and *strB* in the soil community DNA samples. The products for all four of these genes were approximately 500 nucleotides in size, aligning with studies from which these primers were selected (**Table 3**).

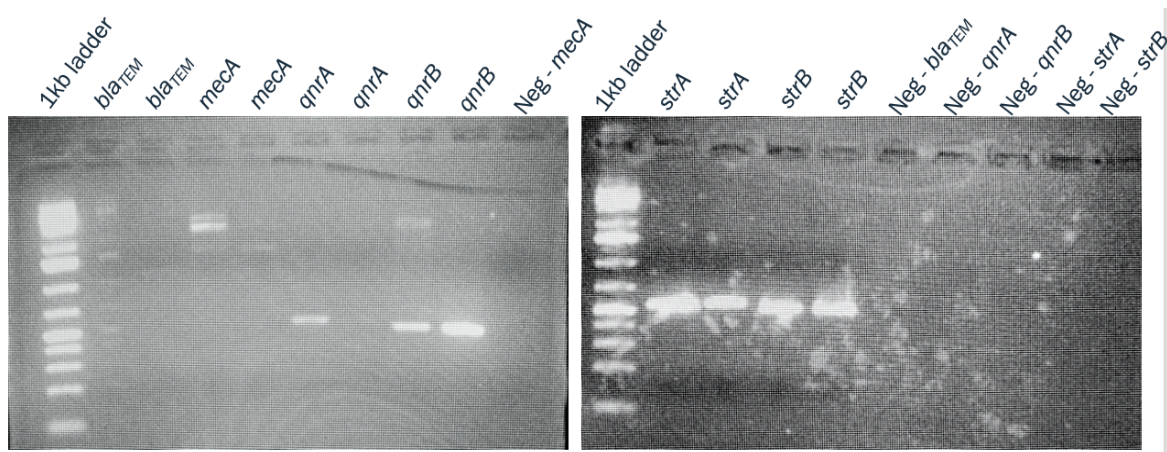


Figure 5. A picture of the PCR products in agarose gel after electrophoresis. Bands for *qnrA*, *qnrB*, *strA*, and *strB* are approximately 500 nucleotides. The lanes designated as “Neg” represent the negative controls for the indicated gene’s primer set. There were no positive controls included.

The gene *qnrB* was then investigated via qPCR to determine the differences in gene copy abundance from the initial time point to the final timepoint. **Figure 6** shows a boxplot comparison of *qnrB* copies between the initial and final timepoints for all donors. Overall there is a significant increase between the initial sampling prior to donor placement and the final

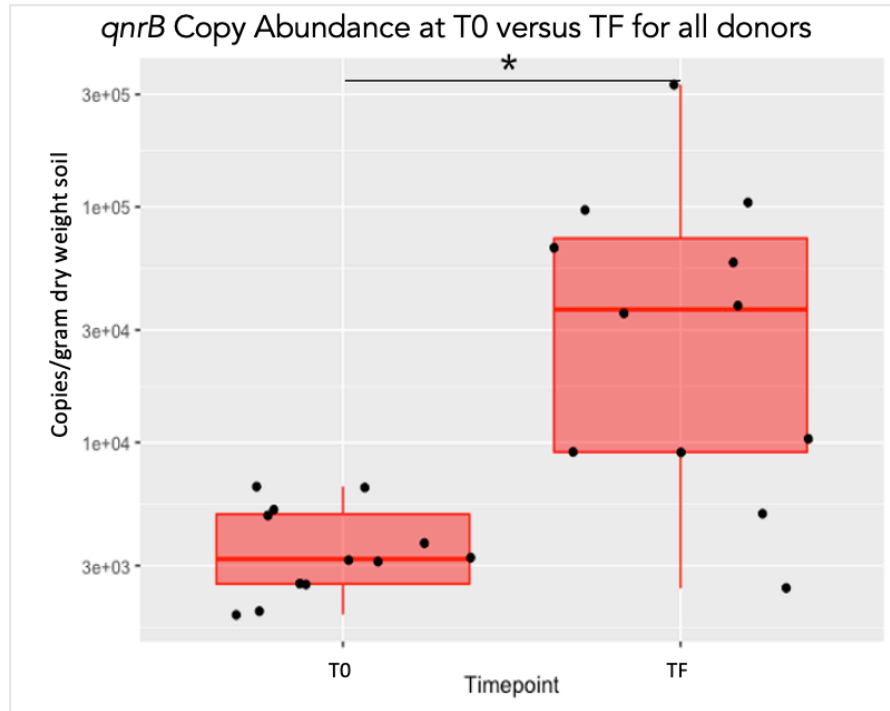


Figure 6. A boxplot comparison of *qnrB* copies from T_0 to T_f for all donors. A * indicates that the difference is significant ($p \leq 0.05$).

sampling at the end of active decay, according to a T-test. The *qnrB* copy abundance between the initial and final timepoints were then compared for each individual donor (**Figure 7**). Ten donors

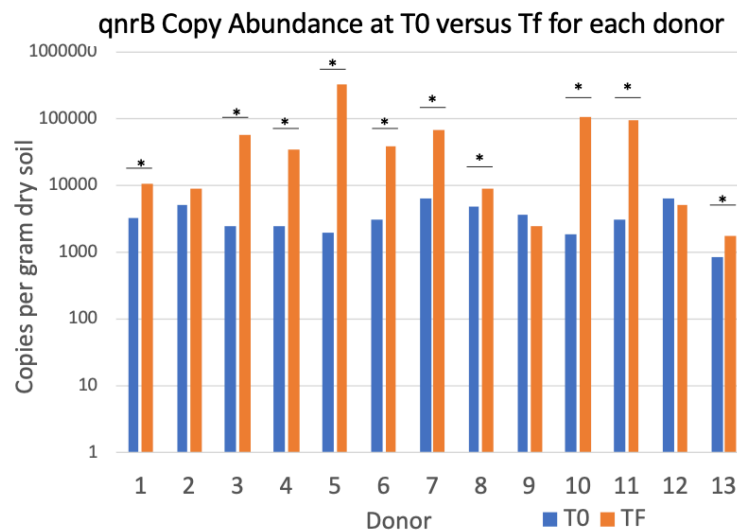


Figure 7. A bar graph comparison of *qnrB* copy abundance from the initial to final timepoints for each donor. A * indicates that the difference is significant ($p \leq 0.05$).

had significant increases in *qnrB* copies. The exceptions to this were the donors TOX002, TOX009, and TOX012. Donors TOX009 and TOX012 had slight decreases in *qnrB* copy abundance from T_0 to T_f , although this decrease was not significant.

Results from the culturing experiment and the qPCR experiment were then compared (Figure 8). A majority of donors had an increase in ciprofloxacin resistant microbes, coinciding with an increase in *qnrB* copies from T_0 to T_f . However, several donors did not have similar results in the two experiments. TOX002 and TOX012 had significant increases in culturable ciprofloxacin-resistant microbes, but they did not have significant increases in *qnrB* copy abundance. In contrast, TOX003 and TOX004 did not have significant increases in ciprofloxacin resistant CFUs, while they did have significant increases in *qnrB* copies. Finally, the number of ciprofloxacin resistant CFUs was compared to the copy number of *qnrB* for all donors at the final timepoint (Figure 9). No relationship was found between these two values.

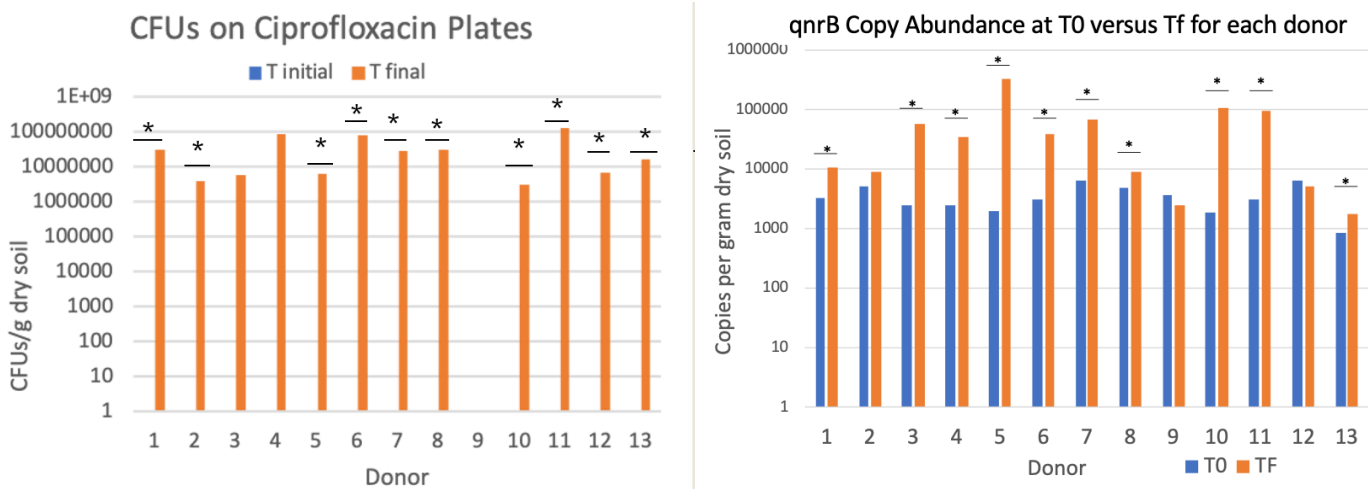


Figure 8. A side-by-side bar graph comparison of the results from the culturing experiment and the qPCR experiment for each donor. A * indicates that the difference is significant ($p \leq 0.05$).

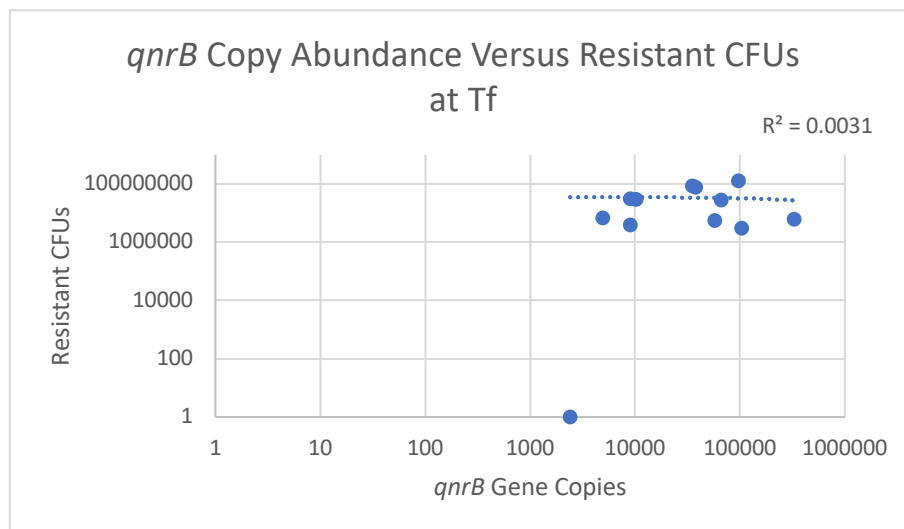


Figure 9. A scatter plot comparison of *qnrB* copy abundance to the number of culturable ciprofloxacin-resistant colonies. The graph represents the final timepoint data for all donors. The R^2 indicates the fit for a linear regression on each graph.

Discussion

Antibiotic resistance (ABR) constitutes a global health and economic threat (CDC 2011). When antibiotic resistance among microorganisms increases, it endangers populations and limits effective pharmaceutical treatment options (Thiele-Bruhn 2003). The increased use of antibiotics in clinical and agricultural settings has led to increased resistance in the human microbiome, particularly in the gastrointestinal tract. During decomposition of cadavers, human-associated microbes, antibiotic residues, and free microbial DNA is transferred to the soil environment. However, it remains unclear how impactful the release of these microbes and molecules are on the overall resistance of soil communities. If decomposition impacts soil communities, the environment can become a reservoir for ABR and can lead to the emergence of new pathogens. These can then be transferred to humans and animals, resulting in novel infections that are difficult to treat. Thus, understanding the relationship between decomposition and antibiotic

resistance is critical as green burials are rising popularity and other countries regularly practice burials without embalming (Mafuvadze 2015, Vatomsky 2018).

This study investigated the changes of antibiotic resistance among soil communities during human decomposition via culture and molecular methods. Comparison of the amount of resistant CFUs between initial and final timepoints indicates that decomposition can impact the ABR of soil microbes, particularly in regard to ampicillin, streptomycin, and ciprofloxacin. Resistance to all three of these drugs increased significantly in the majority of donors studied. However, the increased number of resistant CFUs for some donors can be attributed to an overall increase in microbial populations. Taking that into account, half of the donors studied displayed increased resistance to ampicillin, streptomycin, and ciprofloxacin in the soil community that could not be explained by an overall increase in soil microbial burden. Furthermore, there was not pervasive resistance to these three antibiotics prior to donor placement, although ampicillin resistance has been previously described as wide-spread in soil environments (Esiobu, Armenta et al. 2002, Nan, Jiang et al. 2019).

These three drugs are clinically relevant antibiotics. Ampicillin is a β -lactam antibiotic that targets both Gram-positive and Gram-negative bacteria. It came into use in 1961 and is commonly prescribed to treat infections of the urogenital tract, skin, respiratory tract, ears, nose and throat (Rafailidis, Ioannidou et al. 2007). Five of seven donors with significant increases in ampicillin resistance had a history of respiratory infections and diseases. Streptomycin is an aminoglycoside antibiotic that blocks bacterial protein synthesis and has been used to treat endocarditis and *Mycobacterium* infections since 1944 (Davies, Gilbert et al. 1964). While two donors with significant increases in streptomycin resistance had cardiovascular diseases, no history of *Mycobacterium* infections has been noted for any donors. Ciprofloxacin is a

fluoroquinolone antibiotic that targets a broad range of bacteria. It became commercially available in 1987. It is used to treat many types of infections, including urinary tract infections, sexually-transmitted infections, lower respiratory tract infections, and gastrointestinal tract infections. (Davis, Markham et al. 1996). Four of the donors that had increased resistance to ciprofloxacin had a history of respiratory infections and diseases, while two had histories of gastrointestinal infections. It is notable that there was no resistance to ciprofloxacin prior to placement for any donor, which may be due to its recent introduction antibiotics to the market.

In contrast, this study did find that ABR to tetracycline and sulfamethazine is not generally impacted by human decomposition. This may be attributed to there being high levels of resistance to these drugs in the soil communities prior to donor placement. This is consistent with other studies which have found that tetracycline and sulfa drug resistance is common in the environment. For example, tetracycline resistance has been discovered in water sources and soil environments (Koike, Krapac et al. 2007, Schutzius, Nguyen et al. 2019). Resistance to sulfa drugs has also been observed across a variety of natural environments (Guardabassi and Agerso 2006, Schutzius, Nguyen et al. 2019). This may be attributable to the fact that tetracyclines and sulfonamides are some of the oldest classes of antibiotics, with tetracycline being introduced in 1948, and sula drugs being introduced in 1935 (Gradmann 2008, Nelson and Levy 2011).

Moreover, the morphological variety found on the plate cultures indicates that resistance to the studied antibiotics can be attributed to many taxa. This finding is compatible with previous studies. Resistance to these antibiotics has been described across a wide range of taxonomic diversity, including the genera *Pseudomonas*, *Enterococcus*, *Enterobacter*, *Burkholderia*, *Enterobacteriaceae*, *Acinetobacter*, and *Proteobacteria* (Esiobu, Armenta et al. 2002, Heuer,

Krogerrecklenfort et al. 2002). Therefore, the environment contains an array of antibiotic resistant microorganisms, and these microorganisms comprise many taxonomic classifications.

This study also used PCR to investigate the presence of six antibiotic resistance genes (ARGs) in the soil microbial community: *bla_{TEM}*, *mecA*, *qnrA*, *qnrB*, *strA*, and *strB*. The genes *bla_{TEM}* and *mecA* confer resistance to ampicillin. When visualized under UV light, multiple bands appeared for these genes. This may be due to problems with primer specificity or to multiple forms of the genes in the community DNA sample. There have been a multitude of sequences identified for the gene *bla_{TEM}*, and the primers used in this study were designed to be universal for all *bla_{TEM}* variants (Bertrand, Weill et al. 2006). The gene *mecA* also has several known sequences (Bertrand, Weill et al. 2006). Therefore, the multiplicity of bands visualized in the gel could be explained by various forms of these genes being present in the microbial community of the soil. However, the presence of the genes *qnrA*, *qnrB*, *strA*, and *strB* in soil samples from several donors was confirmed via PCR. The genes *qnrA* and *qnrB* confer resistance to ciprofloxacin, while *strA* and *strB* confer resistance to streptomycin (Srinivasan, Nam et al. 2008, Liu, Yang et al. 2013). Thus, some resistance to each of these antibiotics is likely attributable to the presence of these genes in the community.

qPCR was then used to compare differences in ARG copy abundance from the initial to final timepoints. Only the gene *qnrB* was studied via qPCR. The majority of donors had increased abundances of *qnrB* present from the initial to final timepoints, indicating proliferation of microbes with this gene or possible transfer of the gene between soil microbes. However, the qPCR results and the culturing results do not match for every donor. Donors TOX002 and TOX012 had significant increases in ciprofloxacin resistant CFUs, but they did not have significant increases in *qnrB* copies. This may be the result of the presence of other genes that

confer resistance to ciprofloxacin, like the gene *qnrA*, which was confirmed to be present in soil samples via PCR. Donors TOX003 and TOX004 did not have significant increases in ciprofloxacin resistant CFUs, yet they did have significant increases in *qnrB* copy abundance. This indicates that the *qnrB* gene is being proliferated in the soil community, but there may be mutations or deficiencies in transcription, translation, or protein processing for this gene that prevent it from conferring functional resistance. The complexity of resistance mechanisms is further highlighted by the lack of direct correlation between the presence of the gene and the capacity for resistance.

Overall, this study indicates that human decomposition does impact the resistance of soil microorganisms to several antibiotics, including ampicillin, streptomycin, and ciprofloxacin. Moreover, this study indicates that the soil community is a reservoir for antibiotic resistance to common antibiotics like tetracycline and sulfamethazine. Given that the soil microbiome is a reservoir of resistance, the soil community may be considered a clinically relevant source of novel emergent infections (D'Costa, McGrann et al. 2006).

However, this study was limited by several factors. First, the Anthropological Research Facility (ARF) at the University of Tennessee has been in operation since 1971, and the soil there has been saturated by donor decomposition. A multitude of donors placed on the same soils for many years could have already altered the resistance of the microbial community by releasing host-associated resistant microbes or antibiotic residues into the local environment. Second, there are several limitations to culturing experiments. The soil samples were flash-frozen in liquid nitrogen and stored at -80°C prior to use in culturing experiments. Flash-freezing could have impacted the microbial community, preventing some microbes present from being cultured. Additionally, less than 2% of known microbes can be cultured (Wade 2002). Therefore, there

may be many resistant microbes which were not accounted for by culturing. Third, the changes studied in this investigation were short-term; the long-term effects of decomposition on the antibiotic resistance of soil microbes has yet to be determined. Per one study, some genera like *Bacteroides* have only been shown to persist for a limited amount of time in the soil (Keenan, Emmons et al. 2018). However, other studies have found that free microbial DNA can persist for several days in soil particulate material (Blum, Lorenz et al. 1996). This ability for free DNA to remain stable in soil may allow these changes to persist for longer periods of time. Evidence for this comes from a study which found that soil samples from cemeteries carry antibiotic resistance genes (Abia, Alisoltani et al. 2019).

There are multiple areas for future studies and research improvement. First, these experiments could be replicated with the control soil samples collected in this study and the compared with the results in this investigation. Additionally, these experiments could be replicated with soil samples collected between the initial and final timepoints outlined here to track changes throughout the active decomposition stage. Most importantly, longer-term studies must be conducted to determine the permanence of the effects of decomposition. Enduring changes to the soil microbiome may increase the probability that resistant pathogens will emerge from the soil community and produce novel infections in humans and animals. Finally, the ARF is located adjacent to the Tennessee River. Given that wastewater has been found to contain antibiotics and antibiotic resistant microbes and is a conduit of ABR in the environment, this facility's location by a major water system could increase the resistance of the water environment as well (Bondarczuk, Markowicz et al. 2016).

Conclusion

This data demonstrates that human decomposition does increase the resistance of the soil community to certain antibiotics, in particular, the antibiotics ampicillin, streptomycin, and ciprofloxacin. Meanwhile, for other antibiotics like tetracycline and sulfonamides, resistance is already common in the soil. Moreover, specific resistance gene abundance in the soil microbiome, such as the ciprofloxacin resistance gene *qnrB*, is increased by human decomposition. Overall, these results indicate that human decomposition does impact the antibiotic resistance of soil microbial communities and that soil microbial communities are reservoirs of resistance. The soil resistance reservoir may give rise to new pathogens which are resistant to current antibiotics. Thus, this reservoir is clinically relevant and may suggest what pathogens will become critical in the future. However, additional studies are needed to determine the permanence of the effects of decomposition on the soil community and to investigate other antibiotics which the soil microbiome may have resistance to.

References

- Abia, A. L. K., A. Alisoltani, E. Ubomba-Jaswa and M. A. Dippenaar (2019). "Microbial life beyond the grave: 16S rRNA gene-based metagenomic analysis of bacteria diversity and their functional profiles in cemetery environments." Sci Total Environ **655**: 831-841.
- Agerso, Y. and D. Sandvang (2005). "Class 1 integrons and tetracycline resistance genes in *Alcaligenes*, *Arthrobacter*, and *Pseudomonas* spp. isolated from pigsties and manured soil." Appl Environ Microbiol **71**(12): 7941-7947.
- Bertrand, S., F.-X. Weill, A. Cloeckaert, M. Vrints, E. Mairiaux, K. Praud, K. Dierick, C. Wildemaue, C. Godard, P. Butaye, H. Imberechts, P. A. D. Grimont and J.-M. Collard (2006). "Clonal Emergence of Extended-Spectrum β -Lactamase (CTX-M-2)-Producing *Salmonella enterica* Serovar Virchow Isolates with Reduced Susceptibilities to Ciprofloxacin among Poultry and Humans in Belgium and France (2000 to 2003)." Journal of Clinical Microbiology **44**(8): 2897-2903.
- Binh, C. T., H. Heuer, N. C. Gomes, A. Kotzerke, M. Fulle, B. M. Wilke, M. Schlöter and K. Smalla (2007). "Short-term effects of amoxicillin on bacterial communities in manured soil." FEMS Microbiol Ecol **62**(3): 290-302.

Bondarczuk, K., A. Markowicz and Z. Piotrowska-Seget (2016). "The urgent need for risk assessment on the antibiotic resistance spread via sewage sludge land application." Environ Int **87**: 49-55.

Carter, D. O., D. Yellowlees and M. Tibbett (2007). "Cadaver decomposition in terrestrial ecosystems." Naturwissenschaften **94**(1): 12-24.

CDC (2011). Antimicrobial Resistance Posing Growing Health Threat. CDC and Partners Celebrate World Health Day 2011 to Draw Attention to the Issue. . CDC Online Newsroom, Centers for Disease Control.

Clardy, J., M. A. Fischbach and C. R. Currie (2009). "The natural history of antibiotics." Curr Biol **19**(11): R437-441.

D'Costa, V. M., K. M. McGrann, D. W. Hughes and G. D. Wright (2006). "Sampling the antibiotic resistome." Science **311**(5759): 374-377.

Davies, J., W. Gilbert and L. Gorini (1964). "STREPTOMYCIN, SUPPRESSION, AND THE CODE." Proc Natl Acad Sci U S A **51**(5): 883-890.

Davis, R., A. Markham and J. A. Balfour (1996). "Ciprofloxacin." Drugs **51**(6): 1019-1074.

Esiobu, N., L. Armenta and J. Ike (2002). "Antibiotic resistance in soil and water environments." Int J Environ Health Res **12**(2): 133-144.

Gradmann, C. (2008). "Book Review: The first miracle drugs: how the sulfa drugs transformed medicine." Med Hist **52**(3): 416-417.

Guardabassi, L. and Y. Agerso (2006). "Genes homologous to glycopeptide resistance vanA are widespread in soil microbial communities." FEMS Microbiol Lett **259**(2): 221-225.

Heuer, H., E. Krogerrecklenfort, E. M. Wellington, S. Egan, J. D. van Elsas, L. van Overbeek, J. M. Collard, G. Guillaume, A. D. Karagouni, T. L. Nikolakopoulou and K. Smalla (2002). "Gentamicin resistance genes in environmental bacteria: prevalence and transfer." FEMS Microbiol Ecol **42**(2): 289-302.

Janssen, P. H. (2008). New Cultivation Strategies for Terrestrial Microorganisms. Accessing Uncultivated Microorganisms, American Society of Microbiology.

Keenan, S. W., A. L. Emmons, L. S. Taylor, G. Phillips, A. R. Mason, A. Z. Mundorff, E. C. Bernard, J. Davoren and J. M. DeBruyn (2018). "Spatial impacts of a multi-individual grave on microbial and microfaunal communities and soil biogeochemistry." PLoS One **13**(12): e0208845.

Koike, S., I. G. Krapac, H. D. Oliver, A. C. Yannarell, J. C. Chee-Sanford, R. I. Aminov and R. I. Mackie (2007). "Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3-year period." Appl Environ Microbiol **73**(15): 4813-4823.

Liu, B. T., Q. E. Yang, L. Li, J. Sun, X. P. Liao, L. X. Fang, S. S. Yang, H. Deng and Y. H. Liu (2013). "Dissemination and characterization of plasmids carrying oqxAB-bla CTX-M genes in Escherichia coli isolates from food-producing animals." PLoS One **8**(9): e73947.

Mafuvadze, A. M. a. B. (2015). "The impact of traditional and religious practices on the spread of Ebola in West Africa: time for a strategic shift." The Pan African Medical Journal **22**(1).

Nan, F., J. Jiang, S. Wu, Y. Zhang, J. Qiu, B. Qiao, S. Li and Z. Xin (2019). "A Novel VIII Carboxylesterase with High Hydrolytic Activity Against Ampicillin from a Soil Metagenomic Library." Mol Biotechnol.

Nelson, M. L. and S. B. Levy (2011). "The history of the tetracyclines." Ann N Y Acad Sci **1241**: 17-32.

Nwosu, V. C. (2001). "Antibiotic resistance with particular reference to soil microorganisms." Res Microbiol **152**(5): 421-430.

Perez-Valera, E., M. Kyselkova, E. Ahmed, F. X. J. Sladeczek, M. Goberna and D. Elhottova (2019). "Native soil microorganisms hinder the soil enrichment with antibiotic resistance genes following manure applications." Sci Rep **9**(1): 6760.

Quigley, E. M. (2013). "Gut bacteria in health and disease." Gastroenterol Hepatol (N Y) **9**(9): 560-569.

Rafailidis, P. I., E. N. Ioannidou and M. E. Falagas (2007). "Ampicillin/Sulbactam." Drugs **67**(13): 1829-1849.

Sandegren, L. (2014). "Selection of antibiotic resistance at very low antibiotic concentrations." Ups J Med Sci **119**(2): 103-107.

Schutzius, G., M. Nguyen and T. Navab-Daneshmand (2019). "Antibiotic resistance in fecal sludge and soil in Ho Chi Minh City, Vietnam." Environ Sci Pollut Res Int.

Srinivasan, V., H.-M. Nam, A. A. Sawant, S. I. Headrick, L. T. Nguyen and S. P. Oliver (2008). "Distribution of Tetracycline and Streptomycin Resistance Genes and Class 1 Integrons in Enterobacteriaceae Isolated from Dairy and Nondairy Farm Soils." Microbial Ecology **55**(2): 184-193.

Thiele-Bruhn, S. (2003). "Pharmaceutical antibiotic compounds in soils – a review." Journal of Plant Nutrition and Soil Science **166**(2): 145-167.

Udikovic-Kolic, N., F. Wichmann, N. A. Broderick and J. Handelsman (2014). "Bloom of resident antibiotic-resistant bacteria in soil following manure fertilization." Proc Natl Acad Sci U S A **111**(42): 15202-15207.

Vatomsky, S. (2018). Thinking About Having a ‘Green’ Funeral? Here’s What to Know. The New York Times. New York, New York.

Ventola, C. L. (2015). "The antibiotic resistance crisis: part 1: causes and threats." P T **40**(4): 277-283.

Wade, W. (2002). "Unculturable bacteria--the uncharacterized organisms that cause oral infections." J R Soc Med **95**(2): 81-83.

Zimmermann, M., M. Zimmermann-Kogadeeva, R. Wegmann and A. L. Goodman (2019). "Mapping human microbiome drug metabolism by gut bacteria and their genes." Nature **570**(7762): 462-467.