Title
Distribution, Emergence, Fate and Transport of Antibiotic Resistance Genes in Environmental Compartments: Studies at the Nexus of Human-Environment Interaction

Permalink
https://escholarship.org/uc/item/5852w6pn

Author
Echeverria Palencia, Cristina Maria

Publication Date
2018

Peer reviewed|Thesis/dissertation
Distribution, Emergence, Fate and Transport of Antibiotic Resistance Genes in Environmental Compartments: Studies at the Nexus of Human-Environment Interaction

A dissertation in partial satisfaction of the requirements for the degree Doctor of Philosophy in Civil and Environmental Engineering

by

Cristina María Echeverría Palencia

2018
The failing of current last line of defense antibiotics has raised concerns over the future treatability of common bacterial infections, drawing particular concern for antibiotic resistance genes (ARGs), which confer resistance and can be transferred among bacteria through horizontal gene transfer. ARGs are ubiquitous and rather than readily degrading, can proliferate in the presence of selective and co-selective pressures, as well as persist in the absence of a bacterial host. In this way, ARGs challenge current perceptions of contaminant behavior, with many questions concerning ARG fate and transport still yet to be answered. The four studies reported here explore ARG extent of dissemination, fate and transport, from the framework of prioritizing environmental compartments that may be of public health significance.
The first research chapter presents a survey of environmental ARG levels in California, sampling air, drinking water and soil across 26 public parks in four cities across California, USA. Data are analyzed by two methods and city values are compared to each other as well as to globally reported values. In conducting this study, city to city disparities in ARG levels are shown for the first time, providing insight into possible hotspots for ARG proliferation.

In the second chapter, Confined Animal Feeding Operations (CAFOs) are confirmed as possible sources for airborne ARGs. Air is sampled from areas directly downwind of agricultural activity and compared to locations regarded as “pristine,” or unassociated with agriculture. Antibiotic resistance is studied via culturally dependent and molecular methods, presenting data on ARGs in viable bacteria as well as found extracellularly.

The third research chapter explores commercial garden products as potential sources of ARG content near homes. Here a survey of the ARG content of 30 commercially available garden products is presented and contextualized in relation to branding, third party certifications and environmentally reported ARG quantities.

Lastly, the final research chapter explores the fate and transport of garden product-derived ARGs through microcosm experiments. Here, four garden products are used to supplement an ARG-free receiving soil and temporal profiles are created to determine short term decay and proliferation of ARGs from commercially available inputs.
The dissertation of Cristina María Echeverría Palencia is approved.

Sanjay K. Mohanty
Pamela J. Yeh
Michael K. Stenstrom
Jennifer Ayla Jay, Committee Chair

University of California, Los Angeles
2018
Dedication Page

Para mi familia, siempre.

Dedico este trabajo a mi familia aquí y allá.

A mis hermanos y hermanas. Cada cual es un dolor de cabeza enorme.

Pero valen la pena.

Y a mis papas. En agradecimiento de todos los sacrificios y esfuerzos.

Pasare toda la vida intentando merecerlo.

Gracias.
Chapter 1: Introduction to antibiotic resistance, antibiotic resistance genes, and the role of the environment ................................................................. 1

Chapter 2: Disparate antibiotic resistance gene quantities revealed across four major cities in California: a survey in drinking water, air, and soil at 24 public parks ......................... 8

Abstract ........................................................................................................ 8

Introduction ................................................................................................... 9

Results .......................................................................................................... 11

bla\textsubscript{SHV} .......................................................................................... 11

sul1 ................................................................................................................. 13

erm\textsubscript{F} and erm\textsubscript{B} .................................................................... 14

California ARG quantities in global context .................................................. 15

Inter-gene observations ............................................................................... 16

Statistical Analysis ....................................................................................... 17

Environment and Health ............................................................................ 17

Materials and Methods ............................................................................. 19

Site Selection ............................................................................................... 19

Sample Collection ....................................................................................... 20

DNA Extraction .......................................................................................... 22

qPCR .............................................................................................................. 23

Cataloguing globally reported ARG quantities .......................................... 24

Supporting Information ............................................................................. 32

Methods: Matrix Spike ............................................................................... 36

Inhibition Testing ......................................................................................... 36

References .................................................................................................. 37
Chapter 3: Antibiotic resistance in airborne bacteria near conventional and organic beef cattle farms in California, USA .......................................................... 45

Abstract .................................................................................................................. 45

Introduction ............................................................................................................... 46

Materials and Methods .......................................................................................... 47

Sample site and collection ....................................................................................... 47

DNA extraction ........................................................................................................ 50

qPCR methods ......................................................................................................... 50

Culture work for antibiotic resistant bacteria ......................................................... 52

Assessing antibiotic resistance ................................................................................ 52

Bacterial identification ............................................................................................. 54

Results and Discussion .......................................................................................... 55

ARGs near sites ....................................................................................................... 55

Antibiotic resistance of isolates ............................................................................. 56

Supporting Information .......................................................................................... 66

Chapter 4: Commercially available garden products as potential sources of antibiotic resistance genes—A survey ................................................................. 83

Introduction ............................................................................................................... 83

Materials and Methods .......................................................................................... 85

Soil and Amendment Selection .............................................................................. 85

Sample Collection .................................................................................................... 86

Soil and Amendment Characterization ................................................................. 86

DNA Extraction ........................................................................................................ 87

qPCR ......................................................................................................................... 87

Results and Discussion .......................................................................................... 89
Chapter 5: Environmental Fate of Antibiotic Resistance Genes Introduced through Application of Commercially Available Garden Products

Introduction .................................................................................................................. 109

Materials and Methods ............................................................................................... 110

Soil and Treatment Selection ....................................................................................... 110

Soil and Treatment Preparation .................................................................................. 111

Generating soil composites ....................................................................................... 111

Microcosm assembly ................................................................................................. 112

Microcosm Sampling ................................................................................................. 113

Sampling Conditions ................................................................................................. 114

DNA Extraction ......................................................................................................... 114

qPCR ............................................................................................................................ 115

Results ......................................................................................................................... 117

Amendment selection ............................................................................................... 117

ARG selection .............................................................................................................. 118

ermF ............................................................................................................................. 119

tetL ............................................................................................................................... 121

sul1 ............................................................................................................................... 121

int1 ............................................................................................................................... 123
16s-rRNA ......................................................................................................................... 124
Overall observations........................................................................................................... 125
References......................................................................................................................... 135

Chapter 6 : Summary and Conclusions............................................................................ 141
List of Figures

FIGURE 2-1 | BLASHV AND SUL1 ARG QUANTITIES IN CALIFORNIA SOILS. .......................................................... 28
FIGURE 2-2 | BLASHV AND SUL1 ARG QUANTITIES IN CALIFORNIA TAP WATER.................................................. 29
FIGURE 2-3 | BLASHV AND SUL1 ARG QUANTITIES IN CALIFORNIA AIR. .......................................................... 30
FIGURE 2-4 | ERMF AND ERMB ARG QUANTITIES IN CALIFORNIA SOILS. .......................................................... 31
FIGURE 3-1 | FRACTION OF RESISTANT ISOLATES .................................................................................................. 63
FIGURE 3-2 | AVERAGE NA ISOLATE GROWTH RATES .......................................................................................... 64
FIGURE 3-3 | AVERAGE PCA ISOLATE GROWTH RATES ......................................................................................... 65
FIGURE 4-1 | OMRI CERTIFIED TO NON-CERTIFIED PRODUCTS ........................................................................ 103
FIGURE 5-1 | ARG CONTENT IN EACH SOIL-TREATMENT COMPOSITE AT TIME ZERO. ...................................... 119
FIGURE 5-2 | ERMF COPIES PER GRAM OF GARDEN PRODUCT TREATED SOIL ....................................................... 127
FIGURE 5-3 | TETL COPIES PER GRAM OF GARDEN PRODUCT TREATED SOIL ....................................................... 128
FIGURE 5-4 | SUL1 COPIES PER GRAM OF GARDEN PRODUCT TREATED SOIL ........................................................ 129
FIGURE 5-5 | INT1 COPIES PER GRAM OF GARDEN PRODUCT TREATED SOIL ......................................................... 130
FIGURE 5-6 | ERMF COPIES PER 16S RRNA GENE COPIES .................................................................................... 131
FIGURE 5-7 | TETL COPIES PER 16S RRNA GENE COPIES .................................................................................... 132
FIGURE 5-8 | SUL1 COPIES PER 16S RRNA GENE COPIES .................................................................................... 133
FIGURE 5-9 | INT1 COPIES PER 16S RRNA GENE COPIES .................................................................................... 134
List of Tables

TABLE 2-1 | P-VALUES FROM WELCH’S T-TEST COMPARISONS BETWEEN SOILS IN CALIFORNIA CITIES.....26
TABLE 2-2 | P-VALUES FROM WELCH’S T-TEST COMPARISONS BETWEEN TAP WATER IN CALIFORNIA CITIES..................................................................................................................................................................................27
TABLE 4-1 | QPCR PRIMER SEQUENCES USED.................................................................................................................................98
TABLE 4-2 | GARDEN PRODUCT SAMPLE IDENTIFICATION..................................................................................................................................................................................................................99
TABLE 4-3 | GARDEN PRODUCT CHARACTERISTICS........................................................................................................................................................................................................100
TABLE 4-4 | ARG GENE COPIES PER GRAM OF GARDEN PRODUCT..................................................................................................................................................................................................101
TABLE 4-5 | ARG COPY GENE COPIES PER 16S-RRNA GENE COPIES......................................................................................................................................................................................102
TABLE 5-1 | QPCR PRIMER SEQUENCES USED.................................................................................................................................................................................................116
TABLE 5-2 | GARDEN PRODUCTS SELECTED FOR MICROCOSM ASSEMBLY..........................................................................................118
Acknowledgements

My first thank you goes to Dr. Jennifer Jay. Thank you for helping me open so many doors. For guiding all of the work we’ve completed together and for giving me so much freedom in defining my graduate experience. I’ve never heard you waver in confidence that I could do this and I thank you immensely for that conviction. It has meant so much.

Thank you to my committee for the support they’ve lent me, not only a researcher but as an individual. Dr. Sanjay Mohanty, Dr. Pamela Yeh, and Dr. Michael Stenstrom.

An additional thank you to Dr. Shaily Mahendra for being in my corner since my time as an undergraduate student.

I would like to thank and recognize all of the co-authors that have lent their time, expertise, and labor to the work presented.

Chapter two of this dissertation was co-authored by Vanessa Thulsiraj, Nghi Tran, Cody A. Ericksen, Isabel Melendez, Michael G. Sanchez, Devin Walpert, Tony Yuan, Elizabeth Ficara, Niru Senthilkumar, Fangfang Sun, Renjie Li, Marisol Cira, Demi Gamboa, Heather Haro, Suzanne Paulson, Yifang Zhu, and Jennifer A. Jay.

Chapter three was co-authored by Helen M. Sanchez, Vanessa Thulsiraj, Amy Zimmer-Faust, Ariel Flores, Madeleine Laitz, Gregory Healy, Shaily Mahendra, Suzanne E. Paulson, Yifang Zhu, Jennifer A. Jay

Chapter four was co-authored by Marisol Cira, Ileana Callejas, Karina Jimenez, Rafael Herrera Jr, and Jennifer A. Jay.

Lastly, Chapter five was co-authored by Marisol Cira, Ileana Callejas, Karina Jimenez, Rafael Herrera Jr, Fernando Antunez, and Jennifer A. Jay.

I would like to particularly thank the team of students who worked with me through the completion of the last two chapters presented here. Thank you for trusting me to lead these projects and believing in me. Thank you for wanting to support me so much that you stayed late and came in on weekends more often than you kept to your assigned schedules. You all worked so hard that you were the single largest force pushing me across the finish line. Thank you for listening to my rants. When I was excited, when I was stressed, all of it. For being open to talking to me about things much bigger than work. I truly don’t think that anywhere on this campus or perhaps any campus you would find another group like us—five science and engineering nerds of color doing lab work at record speed while talking about hip hop, relationships, growing up with no money, needing to support our families, guilt for going to school and the migration stories of our parents. Being your full self in a laboratory means everything. Walking through spaces not meant for you as yourself and not as someone trying to
imitate the norm means everything. Thank you for letting me be myself and I truly hope that you all will have the ability bring your authentic selves with you everywhere you go. You are all brilliant. You are all resilient beyond measure. And you are all so deserving of all the doors you are opening for yourselves. If you ever find yourselves in a rut though, know that I’m always here. And I promise I won’t even make you weigh soil while we talk.

Thank you Marisol Cira, Karina Jimenez, Ileana Callejas and Rafael Herrera Jr.

My mentees. Uniqueness of background—ethnicity, race, gender, and social class, among many other things—can be as equally isolating as it is empowering. In my moments of highest vulnerability and self-doubt, you all lifted me through supporting me and trusting me to support you back. Because I think the world of all of you, I found value in being useful along your path. Whether it was a relationship of brief interactions or consistent conversations, I mean it when I say you were my mechanism of retention. Thank you.

The activists. “The movement” has given me far more than I will be able to pay back. In the movement I found a space to develop and friends who have gone on to put my accomplishments to shame with their sheer magnificence. Thank you for shaping my voice. Without knowing it, you prepared me for graduate school and gave me the tools to successfully finish while trying to bring as many people as I could along the way.

The following is a listing of mentees, friends, people I admire and others whose presence in my life was defining prior to and during my graduate studies. Thank you.

Chantiri and Martha. No one will ever understand my graduate school process as much as you two. You’re the people I go to when I want to laugh off all my cynical and sarcastic thoughts. We talk a lot of shit. We really do. I admire you both without bounds and the fact that you both choose to think the same of me is a privilege I don’t feel like I deserve most days. Thank you for being some of the strongest, bravest women I’ve ever had in my life.

Dra. Leisy Abrego. We met right before I started grad school, if I remember correctly. I think you somehow knew that I would need you even though I was too used to standing on my own two feet at that time to know it myself. My father taught me not to make people into role models lightly and I can’t pinpoint exactly when you became one for me, but I’m immensely grateful that you did. Thank you for being kind, gentle, strong, sincere and genuine. For welcoming me and reassuring me through example that it’s important to do things your way. You remaining yourself reminds and challenges me to remain myself.
Fernando Antunez. For being the person I’ve cried to and confided in for the last three years. For learning to sample soil, filter water for microbes and assist in extracting DNA during my last push. And bringing me food. And teaching me to drive. For dropping everything whenever I need you most and making my emergencies your emergencies. For your gentleness, patience, support and solidarity. Thank you for being the best partner I’ve ever had, better than I could ever hope for.

Los Echeverriases y los Palencias. Gracias a mi familia en Guatemala. De la distancia siempre han estado con nosotros. Gracias por el apoyo desde allá.

My siblings. Alejandro, Daniel, Diana, Sofia y Byron. I hope to never forget how much you have all sacrificed so I could get here. If I do, feel free to show me this page of my dissertation and put me in my place. I won’t be sappy here so you don’t make fun of me for years to come.

Mis papás. Soy orgullosamente la hija de Lubia Lileana Palencia Hernandez y Byron Rubén Echeverría Gonzalez. Gracias por regalarme tu paciencia y mi carácter. Y por esperar nada menos de mí. Por dejarme seguir a mis locuras y por consolarme cuando las cosas no salen.

Los quiero mucho.
VITA

Education
2013-2015  M.S., Civil and Environmental Engineering, University of California, Los Angeles
2008-2011  B.S., Chemical and Biomolecular Engineering, University of California, Los Angeles

Publications


Presentations


Poster presentation at the NextProf Fall Engineering Workshop, Ann Arbor, Michigan, October 2016.

Haro, H., **Echeverria, C.**, Jay, J. Identifying Antibiotic Resistant Genes, BlaSHV and Sul1, in the Los Angeles Human Microbiome. Poster presentation at the Southern California Conference for Undergraduate Research, Claremont, California, November 2015.

Haro, H., **Echeverria, C.**, Jay, J. Identifying Antibiotic Resistant Genes, BlaSHV and Sul1, in the Los Angeles Human Microbiome. Oral presentation at the Summer Research initiative, Los Angeles, California, August 2015.


**Project Awards and Grants**
UCLA Law, Animal Law and Policy Small Grants Program, Fall 2017- Spring 2018
Center for Occupational and Environmental Health Student Project Award, Fall 2017-Spring 2018

**Fellowships, Awards and Distinctions**
UCLA 2018 Graduate Student Diversity, Equity and Inclusion Award, Spring 2018
Edward A. Bouchet Graduate Honor Society Inductee, Spring 2018
University of California Presidential Fellowship, Winter, 2018
Hispanic Scholarship Fund (HSF) Scholar, Fall 2017-Spring 2018
Center for Excellence in Engineering and Diversity Leadership Scholarship, Fall 2016-Spring 2017
NextProf Fall Engineering Workshop Participant, Class of 2016
Eugene V. Cota-Robles Fellowship, Fall 2013- Spring 2017
Chapter 1: Introduction to antibiotic resistance, antibiotic resistance genes, and the role of the environment

The increasing failure of antibiotics depended on to treat common bacterial infections has ushered in worry of a post-antibiotic era that would challenge modern medicine as we understand it today. Just this year, a world health report prioritizing the global surveillance of antibiotic consumption cautioned that antibiotic resistance (AR) is a “major threat to health and human development,” calling for a global methodology for data collection. Similarly, the G20, the Obama White House Administration and the U.S.A. Center of Disease Control have issued similar declaration and action plans, highlighting the global scale of urgency associated with increasingly frequent AR.

Antibiotic resistance is mediated at the genetic level through the acquisition and expression of antibiotic resistance genes (ARGs), which “protect an organism from the inhibitory effect of an antimicrobial that is produced by another organism.” ARGs confer resistance through codifying proteins responsible for the detoxification of antibiotics, or efflux pumps. Thus, ARGs provide a key point in potential mitigation and are considered contaminants of emerging concern.

ARGs are attained by microorganisms through the mutation of existing genes or through the acquirement of new genes via horizontal gene transfer (HGT). While historical antimicrobial resistance in the natural environment can occur through both mechanisms, the increase in antibiotic resistance genes globally is believed to primarily be attributed to HGT coupled with unprecedented levels of antimicrobial production and use which exert a selective pressure.
While antibiotics are used for medical reasons, the large majority of antibiotics, are used in agriculture.\textsuperscript{12,13} The adoption of confined animal feeding operations (CAFOs) as a model for the growth of animals intended for human consumption is enabled through use of antibiotics as feed additives both as a method of infection treatment as well as an inducer of growth promotion.\textsuperscript{13} Such antibiotic use accounts for 13.98 million kilograms of antibiotics domestically sold and distributed within the United States (60-80\% of total antimicrobial production).\textsuperscript{12,14} Of these, 60\% are medically relevant.\textsuperscript{14} A significant proportion of antibiotics administered in feed (30-90\%) are excreted in bioactive form and an additional percentage in the form of residues that can be transformed to the original compound post-excretion.\textsuperscript{15} Additionally, consistent exposure to sub-therapeutic doses of antibiotics results in a selective pressure within the microbiome of animals grown for food and subsequent excretion of antibiotic resistant bacteria (ARB). Both antibiotics and ARB from use of the CAFO model are then introduced into the natural and built environment via direct input, airborne transport\textsuperscript{16} and manure application.\textsuperscript{17} Through these mechanisms, they go on to select for and drive ARG proliferation in the environment.

The role the environment in plays in the proliferation of antibiotic resistance clinically continues to be studied, with work pointing to the environment as a key component in the emergence of antibiotic resistance pathogens. Indeed, several clinical isolates are believed to be environmentally derived.\textsuperscript{18-20} In this way, environmental antibiotic resistance is being shown to have a cyclical relationship to clinical antibiotic resistance, creating a great need for monitoring and understanding fate and transport of environmental antibiotic resistance genes to inform future mitigation practices.\textsuperscript{19}

In the studies presented here, the nexus of antibiotic resistance in the environment and humans is explored using a combination of environmental engineering, molecular and
microbiology techniques to better understand ARG extent of dissemination and behavior in compartments relevant to human interaction. We focus on public parks, spaces meant to invite human-environmental interaction, airborne bacteria in areas proximal to CAFOs, and the potential of commercially available, manure-containing gardening products as vectors of ARGs in home gardens and landscaping.

Chapter 2) A regional survey of ARGs in 26 public parks was completed in California, highlighting four major cities of varying urbanization and agriculture scales. Sampling occurred across three environmental compartments—soil, drinking water and air—and 4 ARGs were quantified and catalogued against reported values, contextualizing ARG quantities in California with those reported globally. Here, profiles are shown to be unique across genes and compartments. Additionally, regional stratification is shown, demonstrating that disparate ARG quantities can occur over distances as small as 100 miles.

Chapter 3) The role of confined animal feeding operations as an input source for airborne ARGs in investigated through a comparison of air downwind of “conventional” and “organic” farms. Antibiotic resistance was examined using molecular approached through the quantification of four ARGs via qPCR as well as using culture based methods via disk diffusion and the introduction of a method for high throughput growth rate comparison.
Chapter 4)

A survey of commercially available garden products explores the purchase of manure-utilizing products as a route for ARG introduction. 20 products across four product categories readily available for in-store purchase at major home and garden stores were quantified for three antibiotic resistance genes and compared to values reported regionally. Manure sources are catalogued when available and the third party certifications are explored as predictors of antibiotic resistance in the absence of labeling regulation.

Chapter 5)

Garden products verified for ARG content in Chapter three are used in laboratory microcosms to investigate ARG behavior upon application. One product from each product category verified for ARG content is selected as a treatment with a potting soil found to not contain ARGs of interest used to model a native soil. ARG decay and proliferation behavior of three genes of interest are monitored through destructive sampling over a period of 24 days. Sampling occurs at the surface of each microcosm as well as 1.5in into the soil to determine whether differences in ARG content exist due to direct sun exposure.
References


Chapter 2: Disparate antibiotic resistance gene quantities revealed across four major cities in California: a survey in drinking water, air, and soil at 24 public parks

Abstract

Widespread prevalence of multidrug and pandrug-resistant bacteria has prompted substantial concern over the global dissemination of antibiotic resistance genes (ARGs). Environmental compartments can behave as genetic reservoirs and hotspots, wherein resistance genes can accumulate and be laterally transferred to clinically relevant pathogens. In this work, we explore ARG copy quantities in three environmental media distributed throughout four cities in California and demonstrate that there exist city-to-city disparities in soil and drinking water ARGs. Statistically significant differences in ARGs were identified in soil, where differences in \textit{bla}_{SHV} gene copies were the most striking-- the highest copy numbers were observed in Bakersfield (6.0 \times 10^{-2} \text{copies/16S-rRNA gene copies and 2.6 \times 10^6 \text{copies/g of soil}}, followed by San Diego (1.8 \times 10^{-3} \text{copies/16S-rRNA gene copies and 3.0 \times 10^4 \text{copies/g of soil}}), Fresno (1.8 \times 10^{-5} \text{copies/16S-rRNA gene copies and 8.5 \times 10^2 \text{copies/g of soil}}) and Los Angeles (5.8 \times 10^{-6} \text{copies/16S-rRNA gene copies and 5.6 \times 10^2 \text{copies/g of soil}}). In addition, ARG copy numbers in the air, water, and soil of each city are contextualized in relation to globally reported quantities and illustrate that individual genes are not necessarily predictors for the environmental resistome as a whole.
Introduction

A sustained rise in antimicrobial resistance is predicted to lead to 10 million deaths per year by 2050, with current resistance accounting for an minimum of 700,000 lives lost per year.\textsuperscript{1,2} Concurrently, recent cases of multi- and pandrug-resistant bacteria have raised questions about the future of current last line of defense antibiotics and by extension, the future treatability of common bacterial infections.\textsuperscript{3,4}

Emergence of antibiotic resistant pathogenic bacteria has directed substantial attention toward antibiotic resistance genes (ARGs), which confer resistance and can be transferred among bacteria through horizontal gene transfer (HGT).\textsuperscript{5} Additionally, environmental co-selective pressures can drive antibiotic resistant strain development, promote persistence of ARGs, and result in ARG abundance in the absence of antibiotic selective pressure.\textsuperscript{6–8} Considered environmental contaminants of emerging concern, ARG abundance in environmental compartments warrants increased documentation.\textsuperscript{9–12} Indeed, recent work has pointed to the importance of environmental routes for disease transmission,\textsuperscript{13–15} with several examples of ARGs in clinical isolates believed to be derived from environmental bacteria.\textsuperscript{15}

Possible ARG sources to the environment including wastewater treatment plants,\textsuperscript{16–18} confined animal feeding operations,\textsuperscript{19,20} manure-fertilized fields,\textsuperscript{21} and medical waste streams\textsuperscript{22,23} comprise an intricate system that consistently inputs ARGs into the environment. However, while these sources continue to be explored, the current volume of studies on the extent of dissemination of ARGs in environmental compartments is wanting.

Background ARG quantities in soils have been confirmed as altered and increasing due to anthropogenic antibiotic use\textsuperscript{19,21,24} but there is little research quantitatively reporting them. Instead, data often focus on fold increases due to a particular human activity, such as manure application\textsuperscript{21,25–27} or reclaimed water use,\textsuperscript{28–30} making site-to-site comparisons difficult. Studies
on waterborne ARG quantities are more extensively available and have included surface, ground and coastal waters, drinking water effluent, and tap water, but the large majority of investigation has not involved water systems that directly interact with general populations, leaving open questions concerning ARG exposure. Air is by far the least studied environmental compartment with the majority of research addressing only antibiotic resistant bacteria (ARB). Some quantitative ARG values have been reported, however, these are typically in response to and downwind of a suspected source, rarely concerning ARGs affecting populations outside of immediate feedlot vicinities.

When available, studies reporting ARG quantities in environmental compartments are often difficult or impossible to compare, with researchers reporting results in different units, as well as reporting different genes. Moreover, a broad resistance profile of a particular site is rare, with many studies focused solely on a single environmental compartment. Several public databases and global surveillance projects exist, however, these focus on clinical isolates, rather than ARGs in the environment.

In this study, ARGs were assessed in 26 public parks across four major California cities and three environmental media: soil, drinking water, and air. It was hypothesized unique ARG profiles exist across medium, genes and location, such that when comparing cities within California, city-to-city differences would be identified. Air, water and soil from each site was analyzed for two ARGs, sul1 and blaSHV, as well as the bacterial 16S-rRNA gene. Additionally, all soil samples were analyzed for the ermF, and ermB genes. The sul1 gene was selected for inclusion in this study due to its proposed use as an urbanization marker, its propensity for persisting in the environment, and a high degree of previously recorded data, allowing for ARG quantity contextualization. The blaSHV gene was included in this study due to its close relationship to genes suggested for environmental monitoring. Additionally, along with erm
genes, bla<sub>SHV</sub> gene copy quantities had been shown to be increasing in soils.<sup>24</sup> ermB and ermF have both been proposed as indicators for assessing the antibiotic resistance status of a particular environment.<sup>47</sup> Together, ermB and ermF can shed light on possible differences in genes that are closely related and operate via similar resistance mechanisms.

Through this work we sought to provide a more comprehensive approach to documenting ARG exposure across a given region by introducing a study design that has not been utilized in conjunction with ARG monitoring. Additionally, this study is to our knowledge, the first to contextualize reported ARG quantities in relation to those reported globally.

**Results**

**bla<sub>SHV</sub>**

Gene copies of bla<sub>SHV</sub>, with gene copies defined as the quantity of the target gene present in sample as determined via qPCR, were regularly detected in all soil samples. For bla<sub>SHV</sub> genes copy numbers measured per gram of soil, all city-to-city comparisons with the exception of the Los Angeles-Fresno comparison revealed statistically significant differences (Welch’s t-test p < 0.05, comparing two cities at a time each with n=24) (Table 2-1), with ARG copy numbers highest in Bakersfield (5.1 x 10<sup>5</sup> - 6.9 x 10<sup>6</sup> copies/g) followed by San Diego (7.7 x 10<sup>2</sup> - 9.0 x 10<sup>4</sup> copies/g when detected), and tailed by both Los Angeles and Fresno (3.5 x 10<sup>1</sup> - 2.1 x 10<sup>3</sup> and 1.5 x 10<sup>1</sup> - 6.7 x 10<sup>3</sup> copies/g respectively), as summarized in Figure 2-1. When normalized by 16S-rRNA gene copies, city-to-city differences persisted (Table 2-1), with all relationships now found to statistically differ (p < 0.1). City quantities for bla<sub>SHV</sub> gene copies from highest to lowest were: Bakersfield (1.9 x 10<sup>-2</sup> - 2.2 x 10<sup>-1</sup> copies/16S-rRNA gene copies), San Diego (1.5 x 10<sup>-5</sup> - 1.2 x 10<sup>-2</sup> copies/16S-rRNA gene copies), Fresno (5.1 x 10<sup>-7</sup> - 6.5 x 10<sup>-5</sup> copies/16S-rRNA gene copies) and Los Angeles (8.2 x 10<sup>-7</sup> - 1.6 x 10<sup>-5</sup> copies/16S-rRNA gene copies), as
summarized in Figure 2-1.

Detection of bla\textsubscript{SHV} fluctuated in drinking water samples collected from Los Angeles, Bakersfield and Fresno. In contrast, bla\textsubscript{SHV} was detected in all San Diego drinking water samples as shown in Figure 2-2 (gene copy numbers ranged from $5.5 \times 10^2$ to $10.0 \times 10^3$ copies/L). Gene copy numbers normalized to per liter of drinking water were statistically different when comparing each city ($p < 0.1$) with the exception of an LA-Fresno comparison (Table 2-2). Overall the ranking of gene copy number from highest to lowest was found to be: San Diego, Bakersfield and equivalent lower relative ranking for Los Angeles and Fresno. When gene copy numbers were compared per 16S-rRNA gene copy number (Figure 2-2), all city differences were statistically significant ($p < 0.1$) with the exception of the LA versus Fresno and San Diego versus Bakersfield comparisons (Table 2-2). When viewed in relation to 16S-rRNA gene, two tiers were seen with San Diego and Bakersfield both showing higher gene copy numbers ($2.7 \times 10^2$ - $1.3$ and $5.0 \times 10^2$ - $2.1$ copies/16S-rRNA gene copies respectively) than Los Angeles and Fresno ($9.4 \times 10^5$ - $5.1 \times 10^3$ and $4.0 \times 10^3$ - $8.6 \times 10^2$ copies/16S-rRNA gene copies respectively).

Due to sampling constraints, air was sampled at only two sites per city, with each site sampled in replicates of three to five. The exception to this was Fresno, where three sites were sampled. bla\textsubscript{SHV} copy numbers per liter of air sampled revealed higher bla\textsubscript{SHV} gene copy numbers in Fresno ($0.19$ - $600$ copies/m$^3$), with gene copy numbers in Bakersfield, Los Angeles and San Diego all found to be lower than Fresno gene copy numbers by approximately 50%, but comparable to each other (Figure 2-3). When analyzed per 16S-rRNA gene copy number, Los Angeles gene copy numbers were lowest ($3.8 \times 10^4$ - $9.3 \times 10^3$ copies/16S-rRNA gene copies), with significant overlap among the Fresno, Bakersfield and San Diego sites (Figure 2-3).
sul1

sul1 was consistently detected in soil across all parks and cities studied, with some variability observed to occur within any given park. City-to-city comparisons for sul1 gene copy numbers were found to be statistically different when comparing San Diego parks to those in Fresno (Table 2-1), with higher sul1 gene copy numbers measured in San Diego (p=0.06). When normalized by 16S-rRNA gene copy number, San Diego was found to have higher gene presence than both Fresno and Bakersfield (p < 0.06), with similar gene copy quantities observed in Fresno and Bakersfield (Table 2-1). Los Angeles exhibited the most park-to-park variability (ranging $1.5 \times 10^5$ - $4.0 \times 10^8$ copies/g and $9.0 \times 10^{-4}$ - 2.7 copies/16S-rRNA gene copies) and ARG quantities did not prove to be significantly different from any of the other three cities, regardless of normalization approach. sul1 values in soil were found to vary from $1.1 \times 10^5$ - 2.2 x $10^7$ copies/g to $9.0 \times 10^{-4}$ - 5.1 x $10^{-1}$ copies/16S-rRNA gene copies, with two LA park values yielding particularly high values of $2.0 \times 10^8$ and $4.0 \times 10^8$ copies/g, and 2.6 and 2.7 copies/16S-rRNA gene copies (Figure 2-1).

Presence of sul1 per liter of water sampled was highly variable in San Diego (detected in 30% samples), Bakersfield (detected in 21% of samples) and Fresno (detected in 13% samples), with Fresno having the lowest frequency of gene detection. In contrast, sul1 was detected in 100% (n=18) of Los Angeles drinking water samples. Absolute gene copy numbers of sul1 in San Diego, Bakersfield and Fresno proved too variable to result in statistically significant city-to-city differences when compared to Los Angeles and to each other, as shown in Figure 2-2 and Table 2-2. When analyzing gene copies per copies of 16S-rRNA gene (Figure 2-2), only the LA-Fresno comparison exhibited a statistically significant city difference, with higher gene copy numbers observed in Los Angeles than in Fresno (p = 0.08).

sul1 gene copy numbers in air were found to vary widely within individual cities with
Fresno air measurements ranging from $1.5 \times 10^1$ to $5.3 \times 10^3$ copies/m$^3$, Bakersfield ranging from $1.4 \times 10^2$ to $7.2 \times 10^2$ copies/m$^3$, Los Angeles ranging from $1.6 \times 10^1$ – $1.6 \times 10^2$ copies/m$^3$, and San Diego ranging from $1.7 \times 10^{-2}$ - $2$ copies/m$^3$. Absolute copy numbers per liter of air indicate a higher incidence of $sul$1 gene found in Fresno site 2 when compared to the other three cities (Figure 2-3). After normalizing by 16S-rRNA gene copy number, Fresno site 2 gene copy numbers remained highest, followed by Bakersfield site 1 as a distant second (Figure 2-3).

**ermF and ermB**

Gene copy measurements for both $erm$F and $erm$B were obtained for soil samples, with both genes greatly fluctuating when detectable (Figure 2-4) —$erm$F fluctuated between $3.9 \times 10^0$ and $2.6 \times 10^5$ copies/g and between $1.7 \times 10^{-8}$ copies/16S-rRNA gene and $2.7 \times 10^{-2}$ copies/16S-rRNA gene, while $erm$B fluctuated from $1.1 \times 10^0$ and $8.4 \times 10^3$ copies/g, and between $7.0 \times 10^{-9}$ copies/16S-rRNA gene and $2.7 \times 10^{-4}$ copies/16S-rRNA gene (Figure 2-4). In general, the $erm$F gene had a higher frequency of detection than $erm$B. $erm$F was consistently identified in Los Angeles park soils, with only one triplicate from Site 1 failing to amplify via qPCR. All other cities contained at least one park with confirmed ARG readings obtained for only 1 out of three park samples. Overall, $erm$F detection frequency decreased in the order: Los Angeles, Fresno, San Diego and lastly, Bakersfield. $erm$B was identified much less frequently, with frequency of detection decreasing in the order: Fresno, Bakersfield, San Diego, and finally, Los Angeles. No statistically different city-to-city relationships could be determined for either gene, regardless of normalization technique. These results place $erm$F as above reported values and $erm$B as well below those currently shown in literature.
**California ARG quantities in global context**

Previous reports of *sul*1 per 16S-rRNA gene copies in archived soils, lake and river sediments and environmental soil prior to manure or wastewater application allow this study to place average California soil quantities as generally above those currently documented in Germany, along the Poudre River in Colorado, USA, and in a cornfield in Virginia, USA. However, the range of values seen in this study is wide enough to be inclusive of many values reported in the aforementioned. The highest previously published values identified are those in sediment from Lake Geneva, Switzerland (approx. up to .08 copies/16S-rRNA gene copies). The higher spectrum of values reported from the Lake Geneva site are found to be comparable to the quantities found in this study, although readings for San Diego and Los Angeles exceed the largest reported lake sediment values at several sampling sites. To our knowledge, these values place California *sul*1 gene copy numbers in soil above or comparable to the highest reported values. Absolute copy number was found to rarely be reported for *sul*1 in the soil compartment, with absolute values identified having also been reported as per 16S-rRNA gene copies.

Gene copy numbers of *sul*1 normalized to per 16S-rRNA gene copies in aquatic environmental compartments have been documented in rivers, seas, lakes, bays, reservoirs, reclaimed water, and to a scarce extent, drinking water. Placed within the context of these studies, it can be determined that drinking water ARG gene copy numbers observed in the California cities studied are generally higher than those recorded in lakes, rivers and bays in locations ranging from Manila, to China, and Colorado, among others. Water bodies similar in order of magnitude to cities in California have been recorded in an urban and industrial river, sites along the Baltic Sea, Swiss lakes and in drinking water treated from Lake Taihu, China. However, nearly all drinking water samples obtained from Los Angeles, CA, exceed these reported values by nearly an order of magnitude.
Relative to reporting for *sul1*, information on *blaSHV* is infrequent, making global contextualization difficult. *blaSHV* per 16S-rRNA gene copy numbers have been reported in sediments in Cuba as well as in archived Scottish soils. ARG quantities in Cuban sediment were found to generally be comparable to soil values obtained for Fresno and Los Angeles, with Scottish soil values falling above those in Fresno and Los Angeles but below those in Bakersfield and San Diego. Absolute *blaSHV* values were only found reported once, in Cuban sediments, with values found to be comparable to San Diego.

Although there exists scarce reporting of the *blaSHV* gene copy numbers in water bodies, this study is able to place *blaSHV* drinking water quantities in Los Angeles and Fresno as below those reported in the Baltic Sea and along the Almendares River in Cuba. However, several Bakersfield and nearly all San Diego samples were found to fall within or above the range reported for the Baltic Sea (approx. 500-1000 copies/L). These two cities fell below the grand mean reported in the Almendares River in Cuba (approx. $10^5$ copies/L), but Los Angeles gene copy numbers were generally within its range of seasonal variation (approx. $1.3 \times 10^3 - 3.2 \times 10^5$).

*ermF* copies per 16S-rRNA gene copies were found to occur in the literature in archived Scottish soils, with values far lower than values frequently identified in this study. Other studies reporting *ermF* report non-detect readings. *ermB* values have been reported in sediments in Cuba ($10^{3.7} - 10^{5.4}$ copies/g), with these falling well above those identified in all California cities studied.

**Inter-gene observations**

When analyzing drinking water samples, we observe that San Diego dominates in *blaSHV* presence across the cities studied, with the dominant city shifting to Los Angeles when analyzing the *sul1* gene. This implies that it is ineffective to test for a small number of genes and assume
results to be representative of ARG abundance in general. This is further observed when observing \textit{erm}F and \textit{erm}B soil data. Both genes provide resistance to erythromycin, acting as adenine N-6-methyltransferases and keeping erythromycin from inhibiting aminoacyl translocation. Despite having a similar function and conferring resistance to the same drug, city-to-city detection comparisons reveal that one gene is not necessarily a predictor for the other. This indicates that even genes closely related in function may not be able to behave as indicators for each other.

**Statistical Analysis**

Linear regression and correlation matrices between each gene and population, population density, distance from nearest feedlot operation revealed no direct relationships in either environmental compartment. ARGs across each environmental compartment could not be largely attributed to a particular factor. Instead, a multifaceted set of influences that make ARG quantity prediction greatly complex. In addition, statistical analysis between genes across environmental compartments additionally yielded no direct relationship between genes.

**Environment and Health**

A study of the resistome in human and environmental samples in low-income human habitats indicated ARG sharing between human, animal, and environmental microbial communities, reporting a positive correlation between the proportion of ARG contigs with a mobile genetic element (MGE) and the number of habitats encoding antibiotic resistance proteins, indicating a role for MGEs in environmental transfer.\(^\text{13}\)

It is when analyzing \textit{bla}\textsubscript{SHV} in soils that we see the highest indicator of disparities in ARG quantities across cities. This is pertinent, as it has been suggested that \textit{bla}\textsubscript{SHV} be given a
REScon1 risk categorization, indicating that there exists published evidence that \( \text{bla}_{\text{SHV}} \) poses a substantial risk for resistance dissemination and treatment failure.\(^{59} \) This is further alarming when considered in conjunction with the lack of current reporting for this gene, necessitating the reevaluation current approaches in reporting.

To conclude, this work amalgamates and extends observations reported globally on the quantity of ARGs in drinking water, soil, and air. Our results indicate that there exists a disparity in ARG quantities in the environment across four major cities within California. Additionally, this disparity is observed to be distinct within each environmental compartment as well as for each gene measured, highlighting the importance of testing a collection of ARGs to effectively identify environmental reservoirs and hinting at a complicated set of factors that dictate environmental ARG presence. These results were found to be consistent when gene copy numbers were analyzed by two different and widely used approaches—quantitative gene copy number per mass or volume of compartment as well as per 16S-rRNA gene copy. Future work should include follow up studies to determine whether significant reservoirs for ARGs play the dual role of also being hotspots for HGT.

This paper presents a monitoring approach that if standardized, prompts a more thorough global assessment and reporting of ARG quantities in environmental compartments. Moving forward, future regional assessments should focus on genes categorized as posing a higher risk for being acquired by and conferring resistance to human pathogens and should include data on co-contaminants, soil characterization, MGEs, and microbial community where possible to create a larger database for statistical analysis. In doing so, further trends that might aid our understanding of antibiotic resistance proliferation and the dissemination of ARGs will be more easily identified.
Materials and Methods

Site Selection

Los Angeles, San Diego, Fresno, and Bakersfield were chosen to provide a balance of land uses as well as a range of urbanization. For each city, three of the parks chosen were in the heart of the city while the other three were positioned towards the outskirts. Proximity to major highways, where mixing of environmental influence and vehicular pollution was most likely, was avoided where possible. Priorities for inclusion in the study included accessible soil, presence of a water source and access to a safe and stable air sampling location. A catalogue of parks chosen for inclusion in this study along with population, population density, zip code, degree of urbanization and distance from the nearest feedlot can be found in Supporting Information.

Park urbanization was categorized as high, mid or low based on the number of roads and buildings located within a 500 m radius of the park. Parks whose 500 m radius included ≥ 75 buildings and ≥ 15 roads were categorized as highly urban while parks whose radius included ≤ 50 buildings and ≤ 10 roads were categorized as having low urbanization. Any park that fell between these designations were denoted as having mid-level urbanization. Buildings that fell on the circumference line were included in the count if over half of the building fell within the designated area of interest. This urbanization scheme is a modified urban intensity index as defined by the U.S. Environmental Protection Agency (EPA). A summary of urbanization categorization for each site can be found in Supporting Information.
Sample Collection

Soils

Three meter-squared plots were randomly selected at each park for soil sampling. Top soil was collected in 50 ml sterile falcon tubes by randomly selecting ten points in each plot and dipping the falcon tube to gather topsoil (0 – 2.5 cm), yielding a composite sample representative of the meter squared plot. This was repeated for the remaining two plots, yielding three falcon tubes that would be taken as representative of the site. Rocks and grass were avoided or removed using sterilized plastic scoops. Samples were kept on ice until transported to the laboratory, where tubes were stored at 4 °C prior to processing. For processing, soil tubes were extensively shaken to homogenize the ten points of collection and 0.25 g of each soil was weighed into individual 2mL screw cap tubes preloaded with 1 ± 0.05 g, 0.7mm garnet beads (MO BIO Laboratories, Inc., Carlsbad, CA) before being stored at -20 °C awaiting DNA extraction. Remaining soil was used to conduct moisture content, hydrolysis (soil composition) and loss on ignition analysis (See Supporting Information, Table S 2-2). All soil was processed within a week of sampling. Three samples were collected from each of the 24 parks, resulting in a total of n=72 soil samples.

Tap water

At each site, tap water was collected from either drinking fountains or exterior spigots. All water sources used were publicly accessible and drinking fountains were prioritized whenever possible. Polypropylene plastic bottles used for sampling were washed and treated with 1.2 N HCl overnight before being rinsed three times with milliQ water immediately prior to sample collection. A total of 10 L of sample was collected at each park just prior to returning to the laboratory. Samples took several minutes to obtain and the resulting 10 L were a composite
of water initially exiting each fountain (no flushing) as well as after several minutes of flushing. Samples were kept on ice for the duration of transport and stored at 4 °C until the time of processing. Processing consisted of vacuum-filtering 3 L of sample water through a 47 mm diameter, 0.4 µm pore size polycarbonate filter (EMD Millipore, Billerica MA). After filtration, filters were folded and placed into 2 mL screw cap tubes preloaded with 1 ± 0.05 g of 0.7mm diameter garnet beads (MO BIO Laboratories, Inc., Carlsbad, CA) as required for DNA extraction. Tubes were subsequently stored at -20 °C awaiting DNA extraction. All processing was performed in triplicate and all samples were processed within 48 hours of collection.

Air

Air was collected in as close proximity to parks as possible and not directly in parks due to considerations including park hours, and duration of the sampling trip. Due to time constraints inherent to air sampling, not every park was sampled for air, yielding a smaller n for air than that for soil and tap water. Air was collected at two sites per city, except for Fresno, where samples were collected from three sites. All sampling occurred in the months of January through March, 2015 to ensure that all cities were sampled within the same season, minimizing seasonal variation as a possible cause for city ARG disparities. In total, 24 samples were obtained for tap water and soil and 9 samples were obtained for air. Summary information for sampling duration and sampling window can be found in Supporting Information, Table S 2-3.

Air sampling was conducted using three to five simultaneously operating personal pumps (SKC 224-PCXR4 Aircheck Sampler, SKC Inc. Eighty Four, PA). Operation was set to a flow rate of 2 liters per minute for at least 4 hours and air samples were obtained from a height of approximately 1 m. Longer sampling times were preferable if possible and variance between sampling duration was corrected for during data analysis. 7 mm diameter, 0.4 µm pore size
polycarbonate filters (EMD Millipore, Billerica MA) and 45.72 mm, 1.6 µm glass fiber filters (EMD Millipore, Billerica MA) were used interchangeably, with glass fiber filters manually cut in a sterile environment to a size of 7 mm. A short preliminary experiment conducted prior to sampling concluded that both filter types yielded a comparable quantity of DNA for the same sampling duration upon extraction. Upon sampling completion, all filters were immediately folded and placed in 2 mL screw cap tubes preloaded with 1 ± 0.05 g of 0.7 mm diameter garnet beads (MO BIO Laboratories, Inc., Carlsbad, CA) as specified for DNA extraction. Samples tubes were placed on ice and kept at 4 °C to the extent possible before transport to the laboratory, where they were stored at -20 °C prior to DNA extraction.

**DNA Extraction**

All drinking water and soil DNA extractions were completed using the MoBio PowerFecal DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) within one week of collection. Air samples were extracted within three months of collection. Extractions proceeded per the manufacturer’s guidelines except for the cell lysis step, where bead-beating for 2 mins via a BioSpec MiniBeadbeater-8 (BioSpec Products, Bartlesville, OK) was used in place of vortexing. An additional 2 mL screw cap tube preloaded with 1 ± 0.05 g of 0.7 mm diameter garnet beads (MO BIO Laboratories, Inc., Carlsbad, CA) and absent of sample (extraction blank) was included in every extraction to identify contamination incurred during the extraction process should it occur. Eluted DNA was aliquoted and stored at -20 °C awaiting qPCR analysis. Total DNA concentration was determined using UV absorption via a Nanodrop 2000C (Thermo Scientific, Waltham MA), as were 260/280 absorbance ratios. Gene recovery for samples of similar soil composition extracted as described in this study was calculated via a matrix spike and found to be 83%. For matrix spike methods, see Supporting Information.
All samples were analyzed for ARG abundance of *sul1*, *blaSHV*, and the 16S-rRNA gene, (a total bacteria surrogate measure) via qPCR. Soil samples were additionally analyzed for *ermB* and *ermF* genes. All Assays utilized SYBR Green Master Mix and entailed a 25 µL reaction volume consisting of 12.5 µl of 2X SYBR Green MasterMix (Life Technologies, Grand Island, NY), 1.25 µL of each primer, forward and reverse primer, and at least 2 µL of template DNA, with molecular grade water comprising any remaining reaction volume. Primers used were as developed and validated previously in literature\textsuperscript{24,51} and can be found in Supporting Information, Table S 2-4, along with primer concentrations. Each assay run included a 7-point standard curve positive control, all applicable extraction blanks and a negative control of molecular grade water, with each sample plated in triplicate. All assays were performed in 96-well reaction plates using StepOne Plus (Applied Biosystems). Temperature cycles used can be found in Supporting Information, Table S 2-3 and were as reported previously in literature.\textsuperscript{24,51}

Both soil and water samples were diluted to a concentration of 0.25 ng/µL prior to qPCR to offset inhibition effects as confirmed by well spike and inhibition dilution. While the *sul1* gene assay was found to amplify smoothly for a total mass of 0.5 ng DNA, the *blaSHV*, *ermF*, *ermB* and 16S-rRNA gene assays were modified to contain 2.5 ng in order to properly amplify. Template DNA and molecular grade water volumes were varied as was necessary for each assay. Dilution factors were back calculated during data analysis in order to reverse this effect and obtain per volume or per mass quantification. This dilution process was not found to be necessary for air samples. For well spike and inhibition dilution methods, see Supporting Information.
Target-containing DNA fragments to serve as positive controls were designed using Geneious coupled with NCBI database information and ordered through IDT Technologies. Known concentrations of the designed DNA fragment were run alongside environmental samples, yielding a seven-point standard curve and allowing for quantitation of gene copies. Melt curves were used to further verify correct target gene amplification.

**Cataloguing globally reported ARG quantities**

When assessing reported ARG quantities, only peer-reviewed articles were considered. Literature was confined to those reporting genes corresponding to the present study, where qPCR was used to determine ARG in environmental samples. Studies assessing gene quantities in subsets—i.e. ARB that were cultured and subsequently analyzed via qPCR—were excluded. Included studies reported values for gene count per volume or mass, or a ratio of gene of interest per 16S-rRNA gene. Values used for comparison to the present data set were estimated from plots where necessary, although directly reported values were prioritized when available. Values considered did not include quantities reported following an input of interest, i.e. intentional antibiotic enrichment, reclaimed water use, metals enrichment, etc. It is important to note that due to limited reporting, values presented here are compared to values across different years.

**Acknowledgements**

We thank N. Garrison and C. Cordova for assistance in planning the study, and the UCLA Institute of the Environment and Sustainability for supporting this work as a Senior Practicum Project. Funding was provided by the Natural Resources Defense Council and the UCLA Institute for the Environment and Sustainability. This material is based upon research performed in a collaboratory renovated by the National Science Foundation under Grant No.
0963183, which is an award funded under the American Recovery and Reinvestment Act of 2009 (ARRA).
Table 2-1 | p-values from Welch’s T-test comparisons between soils in California cities

<table>
<thead>
<tr>
<th>Soil: blaSHV</th>
<th>LA (copies/g)</th>
<th>SD (copies/g)</th>
<th>Bks (copies/g)</th>
<th>F (copies/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>0.024</td>
<td>0.003</td>
<td>0.340</td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td>0.003</td>
<td>0.025</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td></td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Soil: sul1</th>
<th>LA (copies/g)</th>
<th>SD (copies/g)</th>
<th>Bks (copies/g)</th>
<th>F (copies/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>0.321</td>
<td>0.313</td>
<td>0.309</td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td>0.186</td>
<td>0.063</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td></td>
<td>0.115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Soil: blaSHV</th>
<th>LA (copies /16S)</th>
<th>SD (copies /16S)</th>
<th>Bks (copies /16S)</th>
<th>F (copies /16S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>0.017</td>
<td>0.003</td>
<td>0.055</td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td></td>
<td>0.003</td>
<td>0.017</td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td></td>
<td></td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Soil: sul1</th>
<th>LA (copies /16S)</th>
<th>SD (copies /16S)</th>
<th>Bks (copies /16S)</th>
<th>F (copies /16S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>0.369</td>
<td>0.284</td>
<td>0.287</td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td></td>
<td>0.05</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td></td>
<td></td>
<td>0.85</td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: 0.05<P-value<0.1  0.01<P-value<0.05  P-value<0.01
### Table 2-2 | p-values from Welch’s T-test comparisons between tap water in California cities

<table>
<thead>
<tr>
<th>Tap Water: blaSHV</th>
<th>LA (copies/L)</th>
<th>SD (copies/L)</th>
<th>Bks (copies/L)</th>
<th>F (copies/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>0.019</td>
<td>0.096</td>
<td>0.370</td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td>0.035</td>
<td>0.019</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td></td>
<td>0.093</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tap Water: blaSHV</th>
<th>LA (copies /16S)</th>
<th>SD (copies /16S)</th>
<th>Bks (copies /16S)</th>
<th>F (copies /16S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>8.74E-05</td>
<td>0.083</td>
<td>0.288</td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td>0.444</td>
<td>8.56E-05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td>0.086</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tap Water: sul1</th>
<th>LA (copies/L)</th>
<th>SD (copies/L)</th>
<th>Bks (copies/L)</th>
<th>F (copies/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>0.347</td>
<td>0.346</td>
<td>0.346</td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td>0.363</td>
<td>0.396</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td></td>
<td>0.739</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tap Water: sul1</th>
<th>LA (copies /16S)</th>
<th>SD (copies /16S)</th>
<th>Bks (copies /16S)</th>
<th>F (copies /16S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>0.126</td>
<td>0.111</td>
<td>0.078</td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td>0.869</td>
<td>0.382</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td></td>
<td>0.409</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Key:**

- 0.05 < P-value ≤ 0.1
- 0.01 < P-value ≤ 0.05
- P-value ≤ 0.01

27
Figure 2-1 | blaSHV and sul1 ARG quantities in California soils.

Soil ARG gene copy numbers across 24 parks in four California cities. Error bars denote intra-park variability when averaged over three sampling triplicates. (a) $bla_{SHV}$ copy numbers normalized to per gram of soil (b) $bla_{SHV}$ copies per 16S-rRNA gene copies (c) $sul1$ copy numbers normalized to per gram of soil (d) $sul1$ copies per 16S-rRNA gene copies
Figure 2-2 | \( \text{bla}_{\text{SHV}} \) and \( \text{sul1} \) ARG quantities in California tap water.

Tap water ARG gene copy numbers across 24 parks in four California cities. Error bars denote intra-park variability when averaged over three sampling triplicates. (a) \( \text{bla}_{\text{SHV}} \) copy numbers normalized to per liter of water (b) \( \text{bla}_{\text{SHV}} \) copies per 16S-rRNA gene copies (c) \( \text{sul1} \) copy numbers normalized to per liter of water (d) \( \text{sul1} \) copies per 16S-rRNA gene copies
Figure 2-3 | blaSHV and sul1 ARG quantities in California air.

ARG gene copy numbers in air across four California cities: (a) bla<sub>SHV</sub> copy numbers normalized to per liter of air (b) bla<sub>SHV</sub> copies per 16S-rRNA gene copies (c) sul1 copy numbers normalized to per liter of air (d) sul1 copies per 16S-rRNA gene copies
Figure 2-4 | ermF and ermB ARG quantities in California soils.

Soil ARG gene copy numbers across 24 parks in four California cities. Error bars denote intra-park variability when averaged over three sampling triplicates. (a) *ermF* copy numbers normalized to per gram of soil (b) *ermF* copies per 16S-rRNA gene copies (c) *ermB* copy numbers normalized to per gram of soil (d) *ermB* copies per 16S-rRNA gene copies
Supporting Information

Table S 2-1 | Sample Site Geographic Characteristics

<table>
<thead>
<tr>
<th>City</th>
<th>Site #</th>
<th>Park Name</th>
<th>Zip Code¹</th>
<th>Population¹ (x10³ people)</th>
<th>Population Density¹ (x10³ people/mi²)</th>
<th>Distance from feedlot¹ (mi)</th>
<th>Urbanization Level²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>1</td>
<td>Will Rogers</td>
<td>90272</td>
<td>22.78</td>
<td>1</td>
<td>155</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Kenneth Hahn</td>
<td>90008</td>
<td>33.28</td>
<td>9.06</td>
<td>67.57</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Seoul</td>
<td>90006</td>
<td>59.5</td>
<td>30.99</td>
<td>63.84</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>MacArthur</td>
<td>90057</td>
<td>45.39</td>
<td>51.26</td>
<td>63</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Grand</td>
<td>90012</td>
<td>29.53</td>
<td>9.13</td>
<td>62.39</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Griffith</td>
<td>90027</td>
<td>45.94</td>
<td>5.56</td>
<td>140</td>
<td>Mid</td>
</tr>
<tr>
<td>San Diego</td>
<td>1</td>
<td>Petco</td>
<td>92101</td>
<td>35.91</td>
<td>7.61</td>
<td>151</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mission Bay</td>
<td>92109</td>
<td>44.01</td>
<td>5.79</td>
<td>152</td>
<td>Mid</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Boone</td>
<td>92114</td>
<td>68.16</td>
<td>8.27</td>
<td>156</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Balboa</td>
<td>92104</td>
<td>45.47</td>
<td>12.01</td>
<td>150</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Torrey Pines</td>
<td>92037</td>
<td>40.11</td>
<td>3.07</td>
<td>145</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Mission Trails</td>
<td>92119</td>
<td>22.97</td>
<td>3.35</td>
<td>150</td>
<td>Low</td>
</tr>
<tr>
<td>Bakersfield</td>
<td>1</td>
<td>Jefferson</td>
<td>93305</td>
<td>37.19</td>
<td>6.27</td>
<td>13</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Deer Peak</td>
<td>93311</td>
<td>42.59</td>
<td>0.25</td>
<td>15</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Patriots</td>
<td>93309</td>
<td>59.49</td>
<td>5.58</td>
<td>12.1</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Bear Mountain</td>
<td>93241</td>
<td>18.54</td>
<td>4.5</td>
<td>1.7</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Tule Elk</td>
<td>93206</td>
<td>2.24</td>
<td>0.01</td>
<td>0.1</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Buena Vista</td>
<td>93268</td>
<td>17.14</td>
<td>0.41</td>
<td>2.5</td>
<td>Low</td>
</tr>
<tr>
<td>Fresno</td>
<td>1</td>
<td>Kearney</td>
<td>93706</td>
<td>38.96</td>
<td>0.24</td>
<td>0.6</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Orchid</td>
<td>93711</td>
<td>37.65</td>
<td>3.32</td>
<td>17</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Fresno Regional Sports Complex</td>
<td>93706</td>
<td>38.96</td>
<td>0.24</td>
<td>0.1</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Pasa Tiempo</td>
<td>93611</td>
<td>47.56</td>
<td>4.25</td>
<td>4.7</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Carozza</td>
<td>93727</td>
<td>70.77</td>
<td>2.18</td>
<td>5.1</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Radio</td>
<td>93703</td>
<td>35.13</td>
<td>7.43</td>
<td>3.8</td>
<td>High</td>
</tr>
</tbody>
</table>

¹ Values as provided by the U.S. census bureau

² Distance and Urbanization designation defined as described in methods
### Table 2-1 | p-values from Welch’s T-test comparisons between soils in California cities

<table>
<thead>
<tr>
<th>Soil: <strong>blaSHV</strong></th>
<th>LA (copies/g)</th>
<th>SD (copies/g)</th>
<th>Bks (copies/g)</th>
<th>F (copies/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>0.024</td>
<td>0.003</td>
<td>0.340</td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td></td>
<td>0.003</td>
<td>0.025</td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td></td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil: <strong>sul1</strong></td>
<td>LA (copies/g)</td>
<td>SD (copies/g)</td>
<td>Bks (copies/g)</td>
<td>F (copies/g)</td>
</tr>
<tr>
<td>Los Angeles</td>
<td>0.321</td>
<td>0.313</td>
<td>0.309</td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td></td>
<td>0.186</td>
<td>0.063</td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td></td>
<td>0.115</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Key:**

- 0.05 < P-value ≤ 0.1
- 0.01 < P-value ≤ 0.05
- P-value ≤ 0.01
Table 2-2 | p-values from Welch’s T-test comparisons between tap water in California cities

<table>
<thead>
<tr>
<th>Tap Water: blaSHV</th>
<th>LA (copies/L)</th>
<th>SD (copies/L)</th>
<th>Bks (copies/L)</th>
<th>F (copies/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>0.019</td>
<td>0.096</td>
<td>0.370</td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td>0.035</td>
<td>0.019</td>
<td>0.093</td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tap Water: sulI</th>
<th>LA (copies/L)</th>
<th>SD (copies/L)</th>
<th>Bks (copies/L)</th>
<th>F (copies/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>0.347</td>
<td>0.346</td>
<td>0.346</td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td>0.363</td>
<td>0.396</td>
<td>0.739</td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: 0.05<P-value≤0.1  0.01<P-value≤0.05  P-value≤0.01
Figure 2-1 | blaSHV and sul1 ARG quantities in California soils.

Soil ARG gene copy numbers across 24 parks in four California cities. Error bars denote intra-park variability when averaged over three sampling triplicates. (a) $bla_{SHV}$ copy numbers normalized to per gram of soil (b) $bla_{SHV}$ copies per 16S-rRNA gene copies (c) $sul1$ copy numbers normalized to per gram of soil (d) $sul1$ copies per 16S-rRNA gene copies.
Figure 2-2 | blaSHV and sul1 ARG quantities in California tap water.

Tap water ARG gene copy numbers across 24 parks in four California cities. Error bars denote intra-park variability when averaged over three sampling triplicates. (a) $bla_{SHV}$ copy numbers normalized to per liter of water (b) $bla_{SHV}$ copies per 16S-rRNA gene copies (c) $sul1$ copy numbers normalized to per liter of water (d) $sul1$ copies per 16S-rRNA gene copies
Figure 2-3 | blaSHV and sul1 ARG quantities in California air.

ARG gene copy numbers in air across four California cities: (a) bla$_{SHV}$ copy numbers normalized to per liter of air (b) bla$_{SHV}$ copies per 16S-rRNA gene copies (c) sul1 copy numbers normalized to per liter of air (d) sul1 copies per 16S-rRNA gene copies.
Figure 2-4 | *ermF* and *ermB* ARG quantities in California soils.

Soil ARG gene copy numbers across 24 parks in four California cities. Error bars denote intra-park variability when averaged over three sampling triplicates. (a) *ermF* copy numbers normalized to per gram of soil (b) *ermF* copies per 16S-rRNA gene copies (c) *ermB* copy numbers normalized to per gram of soil (d) *ermB* copies per 16S-rRNA gene copies
### Table S 2-1 | Sample Site Geographic Characteristics

<table>
<thead>
<tr>
<th>City</th>
<th>Site #</th>
<th>Park Name</th>
<th>Zip Code</th>
<th>Population(^1) (x10(^3) people)</th>
<th>Population Density(^1) (x10(^3) people/mi(^2))</th>
<th>Distance from feedlot(^2) (mi)</th>
<th>Urbanization Level(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Los Angeles</td>
<td>1</td>
<td>Will Rogers</td>
<td>90272</td>
<td>22.78</td>
<td>1</td>
<td>155</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Kenneth Hahn</td>
<td>90008</td>
<td>33.28</td>
<td>9.06</td>
<td>67.57</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Seoul</td>
<td>90006</td>
<td>59.5</td>
<td>30.99</td>
<td>63.84</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>MacArthur</td>
<td>90057</td>
<td>45.39</td>
<td>51.26</td>
<td>63</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Grand</td>
<td>90012</td>
<td>29.53</td>
<td>9.13</td>
<td>62.39</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Griffith</td>
<td>90027</td>
<td>45.94</td>
<td>5.56</td>
<td>140</td>
<td>Mid</td>
</tr>
<tr>
<td>San Diego</td>
<td>1</td>
<td>Petco</td>
<td>92101</td>
<td>35.91</td>
<td>7.61</td>
<td>151</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mission Bay</td>
<td>92109</td>
<td>44.01</td>
<td>5.79</td>
<td>152</td>
<td>Mid</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Boone</td>
<td>92114</td>
<td>68.16</td>
<td>8.27</td>
<td>156</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Balboa</td>
<td>92104</td>
<td>45.47</td>
<td>12.01</td>
<td>150</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Torrey Pines</td>
<td>92037</td>
<td>40.11</td>
<td>3.07</td>
<td>145</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Mission Trails</td>
<td>92119</td>
<td>22.97</td>
<td>3.35</td>
<td>150</td>
<td>Low</td>
</tr>
<tr>
<td>Bakersfield</td>
<td>1</td>
<td>Jefferson</td>
<td>93305</td>
<td>37.19</td>
<td>6.27</td>
<td>13</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Deer Peak</td>
<td>93311</td>
<td>42.59</td>
<td>0.25</td>
<td>15</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Patriots</td>
<td>93309</td>
<td>59.49</td>
<td>5.58</td>
<td>12.1</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Bear Mountain</td>
<td>93241</td>
<td>18.54</td>
<td>4.5</td>
<td>1.7</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Tule Elk</td>
<td>93206</td>
<td>2.24</td>
<td>0.01</td>
<td>0.1</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Buena Vista</td>
<td>93268</td>
<td>17.14</td>
<td>0.41</td>
<td>2.5</td>
<td>Low</td>
</tr>
<tr>
<td>Fresno</td>
<td>1</td>
<td>Kearney</td>
<td>93706</td>
<td>38.96</td>
<td>0.24</td>
<td>0.6</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Orchid</td>
<td>93711</td>
<td>37.65</td>
<td>3.32</td>
<td>17</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Fresno Regional Sports Complex</td>
<td>93706</td>
<td>38.96</td>
<td>0.24</td>
<td>0.1</td>
<td>Low</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Pasa Tiempo</td>
<td>93611</td>
<td>47.56</td>
<td>4.25</td>
<td>4.7</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Carozza</td>
<td>93727</td>
<td>70.77</td>
<td>2.18</td>
<td>5.1</td>
<td>High</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Radio</td>
<td>93703</td>
<td>35.13</td>
<td>7.43</td>
<td>3.8</td>
<td>High</td>
</tr>
</tbody>
</table>

\(^1\) Values as provided by the U.S. census bureau

\(^2\) Distance and Urbanization designation defined as described in methods
<table>
<thead>
<tr>
<th>City</th>
<th>Site #</th>
<th>Soil Composition</th>
<th>Soil Moisture Content (%)</th>
<th>Loss on Ignition Analysis</th>
<th>% Total Solids</th>
<th>% Fixed Solids</th>
<th>% Volatile Solids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% Sand</td>
<td>% Clay</td>
<td>% Silt</td>
<td>% Total Solids</td>
<td>% Fixed Solids</td>
<td>% Volatile Solids</td>
</tr>
<tr>
<td>Los Angeles</td>
<td>1</td>
<td>0.795</td>
<td>0.106</td>
<td>0.100</td>
<td>0.148</td>
<td>0.871</td>
<td>0.890</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.665</td>
<td>0.146</td>
<td>0.190</td>
<td>0.062</td>
<td>0.942</td>
<td>0.887</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.775</td>
<td>0.096</td>
<td>0.130</td>
<td>0.090</td>
<td>0.918</td>
<td>0.963</td>
</tr>
<tr>
<td>San Diego</td>
<td>1</td>
<td>0.735</td>
<td>0.116</td>
<td>0.150</td>
<td>0.061</td>
<td>0.943</td>
<td>0.837</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.725</td>
<td>0.086</td>
<td>0.190</td>
<td>0.170</td>
<td>0.854</td>
<td>0.885</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.615</td>
<td>0.206</td>
<td>0.180</td>
<td>0.218</td>
<td>0.821</td>
<td>0.936</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.775</td>
<td>0.106</td>
<td>0.120</td>
<td>0.082</td>
<td>0.920</td>
<td>0.736</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.755</td>
<td>0.086</td>
<td>0.160</td>
<td>0.147</td>
<td>0.872</td>
<td>0.804</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.865</td>
<td>0.076</td>
<td>0.060</td>
<td>0.012</td>
<td>0.988</td>
<td>0.978</td>
</tr>
<tr>
<td>Bakersfield</td>
<td>1</td>
<td>0.685</td>
<td>0.086</td>
<td>0.230</td>
<td>0.126</td>
<td>0.888</td>
<td>0.935</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.695</td>
<td>0.096</td>
<td>0.210</td>
<td>0.170</td>
<td>0.855</td>
<td>0.935</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.675</td>
<td>0.106</td>
<td>0.220</td>
<td>0.157</td>
<td>0.865</td>
<td>0.954</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.715</td>
<td>0.096</td>
<td>0.190</td>
<td>0.181</td>
<td>0.847</td>
<td>0.935</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.615</td>
<td>0.106</td>
<td>0.280</td>
<td>0.222</td>
<td>0.819</td>
<td>0.908</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.695</td>
<td>0.106</td>
<td>0.200</td>
<td>0.198</td>
<td>0.835</td>
<td>0.938</td>
</tr>
<tr>
<td>Fresno</td>
<td>1</td>
<td>0.455</td>
<td>0.166</td>
<td>0.380</td>
<td>0.161</td>
<td>0.863</td>
<td>0.920</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.675</td>
<td>0.116</td>
<td>0.210</td>
<td>0.169</td>
<td>0.856</td>
<td>0.952</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.615</td>
<td>0.116</td>
<td>0.270</td>
<td>0.203</td>
<td>0.831</td>
<td>0.938</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.685</td>
<td>0.096</td>
<td>0.220</td>
<td>0.227</td>
<td>0.815</td>
<td>0.908</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.685</td>
<td>0.126</td>
<td>0.190</td>
<td>0.047</td>
<td>0.955</td>
<td>0.954</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0.745</td>
<td>0.086</td>
<td>0.170</td>
<td>0.052</td>
<td>0.951</td>
<td>0.881</td>
</tr>
<tr>
<td>City</td>
<td>Site #</td>
<td>Sampling Duration (mins)</td>
<td>Sampling Window</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>--------</td>
<td>--------------------------</td>
<td>--------------------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Los Angeles</td>
<td>1</td>
<td>240</td>
<td>Early morning through early afternoon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>240</td>
<td>Early morning through early afternoon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>San Diego</td>
<td>1</td>
<td>720</td>
<td>Early morning through evening</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>720</td>
<td>Evening through night</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bakersfield</td>
<td>1</td>
<td>705</td>
<td>Early morning through evening</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>540</td>
<td>Early morning through early evening</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresno</td>
<td>1</td>
<td>240</td>
<td>Early morning through early afternoon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>240</td>
<td>Early morning through early afternoon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>240</td>
<td>Early morning through early afternoon</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table S 2-4 | qPCR Primer Sequences Used

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer</th>
<th>Concentration</th>
<th>Sequence (5'→3')</th>
<th>Annealing Conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>16S-rRNA-F</td>
<td>(100) nM</td>
<td>CCTACGGGAGGCAGCAG</td>
<td>60 °C/60 s</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>16S-rRNA-R</td>
<td>(100) nM</td>
<td>ATTACCGCGGTGCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sul1</td>
<td>sul1-F</td>
<td>(200) nM</td>
<td>CGCACCCGAAACATCGCTGCAC</td>
<td>65 °C/60 s</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td>sul1-R</td>
<td>(200) nM</td>
<td>TGAAGTTCCGCGCAAGGCTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>blasIV</td>
<td>blasIV-F</td>
<td>(400) nM</td>
<td>TGATTTATCTGCAGGATACG</td>
<td>55 °C/60 s</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>blasIV-R</td>
<td>(400) nM</td>
<td>TTGACGTTCGATGCTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ermB</td>
<td>ermB-F</td>
<td>(500) nM</td>
<td>AAAACCTACCGCCGACCTACCA</td>
<td>60 °C/30 s</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>ermB-R</td>
<td>(500) nM</td>
<td>TTTGCGGTTCATTGCTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ermF</td>
<td>ermF-F</td>
<td>(500) nM</td>
<td>TCGTTTTACGCGTCAGCATT</td>
<td>60 °C/30 s</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>ermF-R</td>
<td>(500) nM</td>
<td>CAACCCAGTGTGTCGTTT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Methods: Matrix Spike

Percent recovery for the DNA extraction and qPCR process was confirmed via a matrix spike. Soils from Los Angeles (three parks total), exhibiting comparable soil characteristics to those included in this study were spiked with both a high concentration ARG spike (order of $10^8$) as well as a concentration on the scale observed in this study (order of $10^7$). These spiked samples were extracted as described in the methods section with the exception that in addition to the extraction blank, two additional controls of ARG spikes in the absence of the soil matrix were included in DNA extraction. All samples were extracted at the same time and were subsequently run through qPCR for the bla$_{SHV}$ gene as described in the methods section. Percent recovery following DNA extraction and qPCR was found to be 83%.

Inhibition Testing

Inhibition Dilutions and Well Spikes

Extracted DNA was diluted to several concentrations determine whether qPCR reaction inhibition occurred for all mediums. It was found that inhibition played a role in both water and soil matrices and both samples were diluted to concentrations that diluted out inhibitors—0.25 ng/µl. Efficacy of dilution to this concentration was confirmed by choosing two samples from each site at random and plating each sample into 6 qPCR wells. Three wells of each sample were spiked with known standard quantities. qPCR was performed as described in the Methods section and results confirmed no inhibition effects with gene copy numbers consistently being equivalent to the sum of the spike quantity and unspiked sample.
References


(9) Pruden, A.; Pei, R.; Storteboom, H.; Carlson, K. H. Antibiotic resistance genes as


(2), 681–693.


(39) Chapin, A.; Rule, A.; Gibson, K.; Buckley, T.; Schwab, K. Airborne multidrug-resistant


(46) Hall, R. M.; Collis, C. M. Antibiotic resistance in gram-negative bacteria: the role of gene


Chapter 3: Antibiotic resistance in airborne bacteria near conventional and organic beef cattle farms in California, USA

Abstract

Levels of antibiotic resistance genes (ARGs) and the fractions of antibiotic resistant bacteria (ARB) among culturable heterotrophic bacteria were compared in outdoor air near conventional (n = 3) and organic (n = 3) cattle rearing facilities. DNA extracts from filters taken from 18 locations were analyzed by quantitative polymerase chain reaction (qPCR) for five ARGs. At the reference (non-agricultural) site, all genes were below detection. ARGs sul1, blaSHV, erm(B), and blaTEM were more frequently detected and at higher levels (up to 870 copies m⁻³ for blaSHV and 750 copies m⁻³ for sul1) near conventional farms compared to organic locations while the opposite was observed for erm(F) (up to 210 copies m⁻³). Isolates of airborne heterotrophic bacteria (n = 1295) collected from the sites were tested for growth in the presence of six antibiotics. By disk diffusion on a subset of isolates, the fractions of ARB were higher for conventional sites compared to organic farms for penicillin (0.9 versus 0.63), cloxacinil (0.74 versus 0.23), cefoperazone (0.58 versus 0.14), and sulfamethazine (0.50 versus 0.33) for isolates on nutrient agar. All isolates’ ΔA600pres/ΔA600abs were measured for each of the six tested antibiotics; isolates from farms downwind of organic sites had a lower average ΔA600pres/ΔA600abs for most antibiotics. In general, all three analyses used to indicate microbial resistance to antibiotics showed increases in air samples nearby conventional versus organic cattle rearing facilities. Regular surveillance of airborne ARB and ARGs near conventional and organic beef cattle farms is suggested.
Introduction

The rising level of antibiotic resistance is a serious public health issue, posing a global threat to human health (Levy 2004; WHO 2014; Woolhouse and Farrar 2014). The livestock industry is one of the largest consumers of antibiotics (Pruden et al. 2013; Mellon et al. 2001), and the common practice of applying sub-therapeutic dosages of antibiotics to livestock has been shown to foster the development of antibiotic resistant bacteria (ARB) (Levy 1978, van den Bogaard 2001; Levy and Marshall 2004; Price et al. 2007). Antibiotic resistance genes (ARGs) confer antibiotic resistance through various mechanisms and are themselves considered to be emerging contaminants (Pruden et al. 2006; Pruden et al. 2012; Schnoor et al. 2014).

ARB and ARG originating in livestock can reach the general population through multiple pathways: retail meat and poultry (Millman et al. 2013; Price et al. 2005), air downwind of animal feeding operations and transportation vehicles (Gibbs et al. 2006; Green et al. 2006; Rule et al. 2008), indoor air (Chapin et al. 2005), soil following land application (Heuer et al. 2011), and surface and groundwater (Pruden et al. 2012; Pruden 2014).

Recent work by McEachran (2015) shows that downwind air samples of cattle feed yards contained antibiotics, bacteria, and ARGs for tetracycline resistance at increased levels compared to air collected upwind. This important work is the only to our knowledge that specifically investigated ARGs in the vicinity of beef cattle feedlots. Dairy cattle and swine feedlots have been shown to be a source of airborne ARGs for tetracycline resistance (Ling et al. 2013). For ARB, airborne bacteria near and inside
swine CAFO facilities can contaminate surrounding air downwind of the facility (Green et al. 2006) and transport ARB offsite (Gibbs et al. 2006).

Due to increasing concern for the role of environmental compartments in human transmission of antibiotic resistance, it is clear more work on the environmental fate and transport of ARB and ARG is needed. Although cattle farms are prevalent throughout California, the impact of farming practice (conventional or organic) on levels of ARB and ARGs in local air has not been documented. This study collected 1295 isolates from three organic farms and three conventional farms, and tested the hypotheses that: 1) ARGs may be present at differing amounts nearby organic and conventional farms; and 2) there may be differences in growth in antibiotic resistance between bacterial isolates collected from air near conventional and organic beef cattle farms. This study combines molecular techniques and two culture-based methods to determine relative prevalence of ARB among heterotrophic bacteria and levels of ARGs in air samples. Both the standard disk diffusion method and a newly developed screening method involving growth of liquid cultures in the presence and absence of antibiotics were employed to assess resistance.

**Materials and Methods**

**Sample site and collection**

The three conventional beef cattle production sites, or feed yards, were in Kern and Fresno Counties, located in the Central Valley of CA, a highly intensive agricultural region. The three organic production sites were located in Santa Barbara and Ventura Counties. We were not able to locate an organic site in close proximity to the conventional sites.
Six sampling events occurred over the summer of 2013. Each sampling event (n=3) compared a conventional and an organic farm and was conducted during the same week to minimize effects of weather variability. All sampling took place on dates with clear, dry weather and moderate winds. Sample collection occurred within three meters of the edge of the farm; animals were visibly present near the perimeter of each farm when sample collection took place. A more detailed table with information for each farm is provided, Table S 3-1.

The first week of sampling events for conventional (C) and organic (O) farms, respectively, took place on June 25 2013 (C1) and June 28 2013 (O1). The second week consisted of two field events on July 2 2013 (C2) and July 3 2013 (O2), and the third week samples were collected on July 11 2013 (O3) and July 12 2013 (C3). Sampling occurred for each farm between the hours of 11:00 am and 5:30 pm.

At the three conventional sites (C1, C2, C3) and at two of the organic sites (O2, O3), wind was weak for at least part of the time, and direction was variable. Odor was prominent in sites C1, C2, and C3. Under these conditions, plume spread over short distances to a degree (50 m) in all directions is anticipated. At site O1 there was steady wind (>5 mph for whole sampling period); the sampling sites were downwind more than 70% of the time. Conventional sites had higher temperatures than organic sites. Two of the conventional sites (C2 and C3) had very low relative humidity; one site (C1) was well within range with the other organic sites (Table S 3-1). The reference site was on Santa Monica Pier in Santa Monica, CA, when the wind was blowing east off the ocean.

For DNA-based analyses, air samples were collected in triplicate from each collection site using personal pumps (SKC 224-PCXR4 Aircheck Sampler, SKC Inc.)
Eighty Four, PA) at a flow rate of two lpm for approximately four hours, on 47 mm
diameter glass fiber filters (EMD Millipore Glass Fiber Filters without Binders, Catalog
No: APFA 047 00), which capture particles greater than 1.6 µm. Air was sampled at
three locations near each farm and effort was made to be as close as possible to the farm
(within three meters distance from the edge of the farm) without entering private
property. Filters were placed in empty Petri dishes and transported to the laboratory on
ice. Once in the lab, filters were stored at -80°C up to seven months until further
processing for molecular analysis of ARGs.

Samples were also collected for the purpose of capturing viable ARB adjacent to
the farm site (within three meters distance) using a bioaerosol sampler (SKC BioSampler,
SKC Inc. Eighty Four, PA.) with a 15 mL liquid medium of 10% glycerol. A vacuum
pump (SKC Inc. Eighty Four, PA. Cat No. 228-9605) powered by a car battery was
initially set at 12.5 lpm and was checked periodically. After the four-hour sampling
period, the collection liquid was transferred to a 50 ml falcon tube (Fisherbrand) and
transported to the laboratory on ice for same day processing for bacterial isolation.

The source of our weather data was MesoWest http://mesowest.utah.edu/, which
archives data collected by National Weather Service, Air quality Management Districts,
and other sources of high quality met data. Weather sites were within 25 km of the sites,
but in most cases within 15 km, and were chosen with careful consideration of nearby
features that influence flows, such as elevation and close proximity to mountains or the
ocean. For example, in one case the measurement site was near the center of a valley, and
the nearest weather station was at the foot of the nearby mountains; the next closest
station more centered in the valley was chosen.
**DNA extraction**

DNA was extracted from 47 mm glass fiber filters using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc, Carlsbad, CA, Catalog No. 12888-50). While polycarbonate filters are traditionally used for molecular based work, a side-by-side comparison showed comparable DNA yield between polycarbonate and glass fiber filters. Also, side-by-side comparisons of MoBio Power Soil and Gene Rite DNA extraction kits showed comparable performance in terms of both DNA quantity (based on Nanodrop) and quality (based on $A_{260nm}/A_{280nm}$) across both filter types. Glass fiber filters were selected for use because this is the intended filter type for the SKC 224-PCXR4 Aircheck Sampler. Modifications on the DNA extraction protocol were made accordingly. The filters were sliced in half using sterile scalpels, folded and aseptically added into separate extraction beaded tubes containing buffer, and agitated for 2 minutes. A comparison of DNA sample extraction using whole filter versus half was conducted to determine best yield (data not shown). Extracts from each filter half were combined and run through the same spin column in the final stages of the established MoBio protocol, yielding a 100µl total volume which was stored at -20°C for further analysis with quantitative polymerase chain reaction (qPCR).

**qPCR methods**

Levels of ARGs were measured using qPCR (Applied Biosystems) (Table S 3-2). A reaction volume of 25 µL containing 12.5 µL of 1X SYBR® Green Master Mix (Applied Biosystems), 0.5 ng of the template DNA, 1.25 µL (see Table S 3-2 for final
concentrations) of forward and reverse primers and 8 µL molecular grade H₂O was used. Table S 3-2 lists the primers and reaction conditions used in the study. Negative template controls (water and extraction blanks) were included with every run, as was a positive control (sewage).

Melting curve analysis was always conducted to indicate correct positive amplification of target genes. Samples from each farm site and the reference location were analyzed in triplicate and run through a laboratory master standard curve to obtain quantitative values. Triplicate values were averaged for each sample. Detected/Not Quantifiable (DNQ) was assigned when only one of three qPCR wells came up with a Cₜ value of 40 or less. When only two of the wells came up positive, with a Cₜ of 40 or less, the well without a positive response was assigned a Cₜ of 45 and included in the average, so the quantitative measurement would account for the very low third well. Standard deviations were calculated among triplicate qPCR reactions. Data were then converted to m⁻³ values using data on concentration of DNA extracted and sampling time.

All master standard curve sequences were designed to a length greater than 250 base pairs, as was specified by the manufacturer, IDT Technologies. Each sequence was obtained by entering accession numbers found in the literature into the National Center for Biotechnology Information Database. Sequences were designed by selecting a 250+ bp region and testing the first and last 30 base pairs of each sequence using PREMIER Biosoft NetPrimer software, with special attention paid to GC% content, self-dimerization, hairpin formation and cross-dimerization. If the first and last 30 bps were not optimal, a new region was selected and the process repeated until an optimal region
was identified. All sequences were ordered via IDT technologies and re-suspended according to the manufacturer’s instructions.

Culture work for antibiotic resistant bacteria

Isolates were cultured from the Bioaerosol liquid sample collected from each site. For each site, 50 µL of the sample was spread onto both nutrient agar (NA) and plate count agar (PCA) Petri dishes. Two types of media were used to culture a diverse set of chemoheterotrophic bacteria, with NA favoring copiotrophic bacteria. A total of 10 nutrient agar (NA) and 10 plate count agar (PCA) plates were prepared for each of the six farm sites. Inoculated plates were incubated for 48 hours at 25°C. After two days, a subset of randomly selected bacterial colonies (obtained from proportional allocation) were streaked onto new plates (containing the same medium from which the isolate originated) three times consecutively to purify.

The total number of bacterial isolates cultured and tested was at least 100 for each of the plate types (NA and PCA) from each of the six farms. Thus, a total n of 688 NA isolates from conventional farms and total n of 607 isolates from organic farms were analyzed for antibiotic resistance.

Assessing antibiotic resistance

The standard method of testing for antibiotic resistance, disk diffusion, was used to test 145 isolates in triplicate for six antibiotics. In this method, a 50 µL aliquot of each isolate in liquid culture was uniformly spread onto the surface of a Mueller-Hinton agar (MHA) plate with an antiseptic metal spreader to form an even film. Antibiotic paper
disks (6 mm in diameter; BD Diagnostic Systems) were placed on the surface of each seeded MHA plate using a sterile pair of forceps. Plates were incubated at 35°C for 16-18 hours. During incubation, antibiotic agents diffuse outwards, creating regions of inhibition within the microbial lawn. The diameters (mm) of the zones of inhibition were measured by a ruler or caliper. According to the standard method, categorization of an isolate as susceptible, intermediate, or resistant based on the diameter of the inhibition zone (using the CLSI interpretative criteria) requires knowledge of the species of the isolate. However, for environmental samples in which the identities of heterotrophs are unknown, the maximum cut-off concentration listed in the CLSI (corresponding to the minimum cut-off diameter) (Huang et al. 2011; Brown and Balkwill 2009) or other reported value is applied to all heterotrophs for comparison. Fraction of the total isolates growing in the presence of antibiotics is then reported for the consortium. Previous studies applying this method have used a variety of media targeting heterotrophs, including Luria-Bertani (Ghosh and LaPara 2007; Negreanu et al. 2012; Xu et al. 2015; Ramsden et al. 2010), R2A for slow growing organisms (Schwartz et al. 2003, Czekalski et al. 2012), NA (Huang et al. 2011; Czekalksi et al. 2012) for copiotrophs, and a defined medium containing peptone, yeast and glucose (PCA or similar) (Ghosh and LaPara 2007; Brown and Balkwill 2009).

To assess growth in the presence of six antibiotics, all 1295 isolates’ ΔA600_{pres}/ΔA600_{abs} was measured using a high-throughput liquid culture-based method. For this assay, each of the 1295 (688 and 607) purified isolates were inoculated into a microcentrifuge tube consisting of 2 ml of medium broth (NA or PCA) and grown overnight at 25°C while continuously shaking on a Rotor Shaker at level three. A 1:40
dilution of each isolate in liquid culture into broth was used when dispensing into high-throughput 384 well plates (E&K Scientific). Our assay (Figure S 3-1) tested six antibiotics at two concentrations: penicillin (2048 mg L⁻¹, 1024 mg L⁻¹), oxytetracycline (8 mg L⁻¹, 2 mg L⁻¹), cefoperazone (512 mg L⁻¹, 128 mg L⁻¹), amoxicillin (256 mg L⁻¹, 64 mg L⁻¹), cloxicillin (512 mg L⁻¹, 128 mg L⁻¹), sulfamethazine (2048 mg L⁻¹, 1024 mg L⁻¹) (Sigma-Aldrich).

Two wells with no antibiotic were also inoculated so that growth of each isolate in the presence of antibiotic could be compared to that isolate’s growth in the absence of antibiotic. Because each isolate’s growth is being compared to its own growth under different conditions, the assay is appropriate for field studies such as this one where identification is impractical. Absorbance readings of plates at OD 600 at 0, 4, and 24 hours were obtained by using the GloMax® Microplate Multimode Reader (Promega). Each isolate’s ΔA600_{pres}/ΔA600_{abs} over the first 24 hours was calculated. For data processing, if the value was greater than one, a value of one was used to signify no deleterious effect on growth of the antibiotic. If the value was negative (in cases where the A600_{pres} decreased, indicating no growth at all in the presence of antibiotic) a value of 0 was used.

**Bacterial identification**

16S rDNA identification was tested on a subset of PCA and NA to compare similarities in microbial communities among farm sites sampled. 17 isolates from PCA were analyzed from each of the two production types: conventional and organic. 11 isolates from NA were analyzed from each of the two production types: conventional and
organic. At least five isolates from each farm were selected. A previously established DNA extraction protocol (Shanks et al. 2012) was used to extract DNA from all isolates. Afterwards, polymerase chain reaction (PCR) was used to amplify the 16S rDNA of the isolates, followed by a purification step with the MoBio 12500-50 UltraClean PCR Clean-Up kit. Gel electrophoresis was used on a selection of samples to verify good quality of PCR product. In addition, DNA quantity and quality was assessed using UV absorption with a Nanodrop 2000C (Thermo Scientific, Waltham MA). Further processing and sequencing of 16S genes was performed at UCLA Genotyping and Sequencing Core (GenoSeq, Los Angeles, CA). Sequences were then blasted using NCBI Blasting website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the bacteria.

**Results and Discussion**

**ARGs near sites**

ARGs, now considered emerging contaminants, have been recently observed in environmental samples, including surface waters (Stoll et al. 2012; Storteboom et al. 2010), recycled water (Fahrenfeld et al. 2013), agricultural sites (Knapp 2010; Knapp et al. 2010; Garder et al. 2014), and river sediments Graham et al. 2011; Su et al. 2014). However, there is a paucity of data on ARB (Gibbs et al. 2006) and ARGs (Ling et al. 2013; McEachran et al. 2015) in air near CAFOs. This study provides the first data on levels of ARGs in air near both conventional and organic of beef production.

We chose to study ARGs *bla*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *sul1*, *erm*(B) and *erm*(F) as they have been previously observed in environmental samples (Knapp 2010; Knapp et al. 2010). Macrolides including tylosin and erythromycin, resistance to which is encoded by *erm*(B)
and *erm*(F), are used in the cattle industry (Burrows et al. 1989; Silbergeld et al. 2008), and penicillins are used in corn ethanol production (the waste product of which, distillers grains, are often fed to cattle (Olmstead 2012)).

Bioaerosol samples collected within three meters distance from the perimeter of each farm were analyzed for five ARGs (Table S 3-2). The non-agricultural reference site showed non-detects for all five genes. ARGs *sul1*, *blaSHV*, *erm*(B), and *blaTEM* were more frequently detected and at higher levels (up to 870 copies m$^{-3}$ for *blaSHV* and 750 copies m$^{-3}$ for *sul1*) near conventional farms compared to organic locations, while for *erm*(F), levels were higher at an organic site (up to 210 copies m$^{-3}$). *erm*(B) and *blaTEM* were only detected at one conventional site each. While there are no reported values in the literature for air samples for the ARGs tested here, these reported values are on the order of those observed for ARGs *tet*(X) and *tet*(W) by Ling et al. (2013) for dairy and swine concentrated animal feeding operations. McEachran et al. (2015) analyzed relative differences in the levels of genes encoding tetracycline resistance between air samples upwind and downwind of beef cattle feed yards and found increased ARG levels downwind by a factor of up to three orders of magnitude.

**Antibiotic resistance of isolates**

Over the past 70 years, food animal production, especially in the U.S., has transformed from small scale to large industrial scale production (Graham et al. 2008). Processes in these conventional farming concentrated animal feeding operations (CAFOs) include breeding, feeding, and animal husbandry, all of which may involve antibiotic use for therapeutic and non-therapeutic purposes. In contrast, organic farms
within the U.S. require cattle to be raised without antibiotics or hormones, and feed must be certified as pesticide and antibiotic-free by the U.S. Department of Agriculture (2012). Organic farms typically provide more access to free range grazing, sunlight, fresh air, and freedom of movement than their CAFO counterparts (USDA website; USDA, 2012).

An important distinction between “conventional” and “organic” farming practices is the known use of antibiotic-enriched feed in the former. Antibiotics are granularly premixed into feed or solubilized into drinking water, aiding in beef cattle weight gain and increased feed conversion efficiency. Another popular feed for domestic livestock is distillers’ grain solubles, a byproduct of ethanol production from corn. Antibiotics including penicillin, erythromycin, tylosin, and virginiamycin are added in the production of distillers’ grain solubles to prevent overgrowth of bacteria in ethanol grain reactors (Olmstead 2012). There is some evidence that drying inactivates the antibiotics, but no data are available on ARB and ARG in this feed (Olmstead 2012).

A subset of isolates from each group was analyzed by the standard disk diffusion method (Figure 3-1). Because this study involved unknown isolates from field samples, the maximum concentration (corresponding to the smallest diameter) reported in the CLSI was used to categorize bacteria as resistant. The ratio of isolates growing in the presence of antibiotic to the total culturable heterotrophs for each type of media was reported as fraction resistant (Ghosh and LaPara 2007; Negreanu et al. 2012, Xu et al. 2015; Ramsden et al. 2010; Schwartz et al. 2003; Czechalski et al. 2012; Huang et al. 2011; Brown and Balkwill 2009).

For NA isolates, the fraction resistant to penicillin was 0.90 (standard error (SE) 0.046) and 0.63 (SE 0.088) for isolates from conventional and organic farms,
respectively. For cloxicillin, the same fractions were 0.74 (SE 0.067) and 0.23 (SE 0.077). For PCA isolates, the fractions resistant to penicillin were 0.97 (SE 0.028) and 0.72 (SE 0.079) for isolates from conventional and organic farms, respectively. For cloxicillin, the same fractions were 0.97 (SE 0.030) and 0.71 (SE 0.080). The one case where the organic farms had a significantly higher resistant fraction was for amoxicillin for the NA isolates only; notably, all of the isolates classified as resistant were from one organic farm that does use antibiotics therapeutically.

A total of 1295 bacterial isolates from locations adjacent to six farms (n > 200 for each farm) were cultured and were tested for growth in the presence of antibiotics. For each farm, the average of each isolate’s ΔA600\text{pres}/ΔA600\text{abs} for each antibiotic was calculated. In general, conventional beef cattle production locations consistently showed a greater average ΔA600\text{pres}/ΔA600\text{abs} compared to organic production (Figure 3-2 and Figure 3-3). Remarkably, farms of each type tended to have similar values for average ΔA600\text{pres}/ΔA600\text{abs} (see error bars, which are +/- one standard deviation on the mean).

For NA, the mean for the three farms of the average ΔA600\text{pres}/ΔA600\text{abs} of all isolates for that farm for conventional and organic, respectively, for each antibiotic (low concentrations) were determined to be as follows: penicillin: 0.86 (SD 0.07) and 0.61 (SD 0.02), cloxicillin: 0.79 (SD 0.08) and 0.60 (SD 0.05), oxytetracycline: 0.76 (SD 0.03) and 0.57 (SD 0.07), cefoperazone: 0.78 (SD 0.19) and 0.61 (SD 0.04), amoxicillin: 0.70 (SD 0.20) and 0.62 (SD 0.08), sulfamethazine: 0.77 (SD 0.08) and 0.60 (SD 0.04) (Figure 3-2). A Welch t-test was done on conventional farms (n=3) versus organic (n=3) for each antibiotic for NA medium. Penicillin (p=0.01), cloxicillin (p=0.02), sulfamethazine (p=0.03), and oxytetracycline (p=0.02), all showed significant differences between
resistance ratios for conventional and organic sites. Results for high antibiotic concentrations also showed generally higher fraction of isolates resistant, notably for penicillin (p<0.00), cloxicillin (p=0.06), and sulfamethazine (p=0.08). (Figure 3-2).

For PCA medium at lower concentrations, the mean for the three farms of the average $\Delta A_{600, \text{pres}}/\Delta A_{600, \text{abs}}$ of all isolates for that farm for conventional and organic were determined to be as follows: penicillin: 0.77 (SD 0.26) and 0.60 (SD 0.21), cloxicillin: 0.79 (SD 0.21) and 0.59 (SD 0.16), oxytetracycline: 0.85 (SD 0.09) and 0.67 (SD 0.03), cefoperazone: 0.88 (SD 0.08) and 0.57 (SD 0.15), amoxicillin: 0.78 (SD 0.14) and 0.60 (SD 0.16), sulfamethazine: 0.82 (0.17) and 0.61 (SD 0.04) (Figure 3-3). A Welch t-test was done on conventional verse organic for each antibiotic for PCA medium. Cefoperazone (p=0.03) and oxytetracycline (p=0.05) showed a significant difference between resistance ratios for conventional and organic sites at the low concentration. At high concentrations, the pattern is similar but not statistically significant for any antibiotic.

At least one antibiotic from each antibiotic class tested showed more resistance in conventional farming practices than in organic. Penicillin and cloxicillin (penicillin group), oxytetracycline (tetracyclines), cefoperazone (cephalosporins) and sulfamethazine (sulfonamides) showed greater resistance in conventional farming. Isolates exposed to higher antibiotic concentration showed the same antimicrobial resistance pattern but showed more variability in the conventional farms.

A randomly selected subset of isolates (n = 64) cultured on either PCA or NA from conventional and organic sites was identified using 16S rDNA sequencing. For all
four groups, *Bacillus* was most prevalent (36-55%), and *Enterobacter* was next highest in prevalence. Detailed information is given in Supplemental Information.

Conventional and organic farming practices have previously been compared with respect to ARB for dairy cattle (Sato et al. 2006; Halbert et al. 2006; Ray et al. 2006), poultry (Luangtongkum et al. 2006; Alali et al. 2010; Heuer et al. 2001; Wittwer et al. 2005; Miranda et al. 2008), and swine (Gebreyes et al. 2005; Mathew et al. 2001; Bunner et al. 2007). Information on resistance to antibiotics is variable; in general, increased resistance has been shown in dairy, poultry and swine but not for beef, where few data are available. Reinstein et al. (2009) reported no systematic difference in antibiotic resistance profiles isolates of *E. coli* O157:H7 from fecal samples from organic, naturally-raised, and conventional beef cattle.

In this study, as tested by both the disk diffusion (Figure 3-1) and the HT (Figure 3-2 and Figure 3-3) method, growth in the presence of antibiotics in airborne isolates was significantly greater for conventional isolates for at least one antibiotic from each class of antibiotics tested: the β-lactams group, which acts by inhibiting synthesis of the peptidoglycan layer of bacterial cell walls (i.e. penicillin, amoxicillin, cloxicillin, and cefoperazone), the tetracycline group (i.e. oxytetracycline), which inhibits protein synthesis by preventing attachment of tRNA to the ribosomal attachment site, and the sulfonamides, which are analogs of para-aminobenzoic acid (PABA), inhibiting DNA synthesis by disrupting normal utilization of PABA (i.e. sulfamethazine).

The World Health Organization (2014) stated that there is a need for increased and improved surveillance of antibiotic resistance and dissemination of data. Only a limited number of countries have surveillance programs for production animals such as
Canada, Denmark, Germany, Japan, Netherlands, and the U.S. Recently, in December 2013, the United States FDA proposed out a voluntary road map plan for industry (FDA 2013) to revise and phase out certain antibiotics from food animal production. Whereas the U.S. holds less stringent policies, other countries such as Denmark have held stronger regulations in place to monitor antibiotic resistance. The Center for Disease Control’s report (2013) found that the link between antibiotic use in animal production and AR infections in humans calls for use of antibiotic use under veterinary oversight, not for growth promotion.

This study expands upon current monitoring approaches by combining both culture-based methods and molecular techniques to determine presence of ARB and ARGs from airborne bacteria. Molecular assays were optimized for detection of ARGs from air samples. This new sampling approach provides a unique framework for future studies that aim to test for presence of airborne ARB and ARGs. The high throughput method may be a useful addition to current tools in antibiotic resistance testing, especially when isolate identity is not known, as each isolate’s growth in the presence of antibiotic is compared to the same isolate’s growth in the absence of antibiotic. The high-throughput antibiotic resistance screening method: 1) allows for analysis of 12 isolates at two levels of an antibiotic in one assay 2) can obtain results in as little as 4 hours (depending on growth rate of organisms and media used) 3) can test multiple media to capture greater diversity of microbial community 4) allows for analysis of six different antibiotics at two concentrations in one assay.

One limitation of this study is that production sites could differ in ARB and ARG because of possible differences in animal population density between sites; however, this
difference between groups was unavoidable as it is inherent in the farming types. Also, as the organic farms were not located in the same general area as the conventional farms, the patterns could purely be climactic, or owing to some other industry in the area. An upwind-downwind study would be ideal, but we found accessibility to sampling locations to be prohibitive to that study design.

All three sets of results from this study suggest that the use of antimicrobials on farms can influence the bacterial resistance patterns depending on beef production type, conventional or organic farming. Future research on the fate of ARB and ARGs in the environment is warranted, including regular monitoring and surveillance of ARB and ARGs from cattle sources.

**Acknowledgements**

This material is based upon research performed in a renovated collaboratory by the National Science Foundation under Grant No. 0963183, which is an award funded under the American Recovery and Reinvestment Act of 2009 (ARRA). We are grateful to Winston Lee, Karmina Padgett, Elizabeth Roswell, Cindy Xiong, and Alicia Amundson. Funding was provided by the Natural Resources Defense Fund and the Institute of the Environment and Sustainability at UCLA.
Figure 3-1 | Fraction of resistant isolates

Bars depict the fraction of resistant isolates for NA (panel A) and PCA (panel B) media for the disk diffusion method for each antibiotic. Resistance was determined using minimum diameters reported in the CLSI. Isolates from three conventional (hollow bars) and three organic farms (filled bars) were tested.
Bars depict the average and standard deviation of the mean growth rate ratio for three farms of each type (n = 6 farms). Each of the 676 NA isolates was grown in liquid culture in the presence and absence of antibiotic (panel A and B depict results for low and high concentrations, respectively) and absorbance was measured at time points. Hollow and filled bars represent the conventional and organic farms, respectively.
Figure 3-3 | Average PCA isolate growth rates

Bars depict the average and standard deviation of the mean growth rate ratio for three farms of each type (n = 6 farms) for the PCA isolates. Each of the 619 isolates was grown in liquid culture in the presence and absence of antibiotic (panel A and B depict results for low and high concentrations, respectively) and absorbance was measured at time points. Hollow and filled bars represent the conventional and organic farms, respectively.
Supporting Information

Table S 3-1 | Meteorological data for conventional sites (C1, C2, C3) and organic sites (O1, O2, O3)

<table>
<thead>
<tr>
<th>Loc.</th>
<th>Date</th>
<th>Wind Speed (MPH), Wind Direction</th>
<th>T (°C) range</th>
<th>RH (%) range</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>O1</td>
<td>6/28</td>
<td>6 – 15, WNW-NE</td>
<td>30 – 32</td>
<td>30-25</td>
<td>Perfect wind direction for sampling</td>
</tr>
<tr>
<td>O2</td>
<td>7/3</td>
<td>0 – 13, 0 – 4 WSW-SW-,SWW; brief stints of WNW-NE sites agree</td>
<td>20.5 – 22</td>
<td>83 – 70</td>
<td>Location is between 2 met stations with steep gradient. Some periods with no wind recorded, which means variable/wind speed too low.</td>
</tr>
<tr>
<td>O3</td>
<td>7/11</td>
<td>0 – 11, 113-315° (ESE, S, SW, WSW, WNW, NW)</td>
<td>24 – 32</td>
<td>58 – 39</td>
<td>Location is between 2 cities, but at same elevation.</td>
</tr>
<tr>
<td>C1</td>
<td>6/25</td>
<td>0 - 5, S-E-N; mostly ESE°</td>
<td>26 – 33</td>
<td>60 – 37</td>
<td>Weak winds, some zero</td>
</tr>
<tr>
<td>C2</td>
<td>7/2</td>
<td>0 - 6 0 + WNW - NNE</td>
<td>39 - 42</td>
<td>17 - 13</td>
<td>Station is near center of valley, slightly south of monitoring sites.</td>
</tr>
<tr>
<td>C3</td>
<td>7/12</td>
<td>0 – 6 0 + SSW, NW - NE</td>
<td>28 - 37</td>
<td>20 - 15</td>
<td>Station is near center of valley, slightly south of monitoring sites.</td>
</tr>
</tbody>
</table>
Table S 3-2 | The primer sequences and qPCR reaction conditions used in the study

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Concentration</th>
<th>Sequence (5’-3’)</th>
<th>Annealing Conditions</th>
<th>Elongation Conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>(erm) (F)</td>
<td>ErmF-F</td>
<td>500 nM</td>
<td>TCG TTT TAC GGG TCA GCA CTT</td>
<td>60°C/30 s</td>
<td></td>
<td>(Knapp)</td>
</tr>
<tr>
<td></td>
<td>ErmF-R</td>
<td>500 nM</td>
<td>CAA CCA AAG CTG TGT CGT TT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(bla_{TEM})</td>
<td>BlaTEM-F</td>
<td>400 nM</td>
<td>TCG GGG AAA TGT GCG</td>
<td>50°C/60 s</td>
<td>72°C/60s</td>
<td>(Knapp)</td>
</tr>
<tr>
<td></td>
<td>BlaTEM-R</td>
<td>400 nM</td>
<td>GGA ATA AGG GCG ACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(bla_{SHV})</td>
<td>BlaSHV-F</td>
<td>400 nM</td>
<td>TGA TTT ATC TGC GGG ATA CG</td>
<td>55°C/60 s</td>
<td>76°C/30s</td>
<td>(Knapp)</td>
</tr>
<tr>
<td></td>
<td>BlaSHV-R</td>
<td>400 nM</td>
<td>TTA GCG TTG CCA GTG CTC G</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(sul)</td>
<td>Sul1-F</td>
<td>250nM</td>
<td>CGC ACC GGA AAC ATC GCT GCA C</td>
<td>65°C /30s</td>
<td>72°C /30s</td>
<td>(Ji)</td>
</tr>
<tr>
<td></td>
<td>Sul1-R</td>
<td>250nM</td>
<td>TGA AGT TCC GCC GCA AGG CTC G</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table S 3-3 | Results from 16S identification of a subset of isolates

<table>
<thead>
<tr>
<th>Type of medium</th>
<th>Type of farm</th>
<th>Identification results</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCA</td>
<td>Conventional (n=28)</td>
<td>36% <strong>Bacillus</strong>, 29% <strong>Enterobacter</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>29% <strong>Staphylococcus</strong>, 3% <strong>Paenibacillus</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3% <strong>Lactobacillus</strong>.</td>
</tr>
<tr>
<td></td>
<td>Organic (n=17)</td>
<td>41% <strong>Bacillus</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>35% <strong>Enterobacter</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>12% <strong>Staphylococcus</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6% <strong>Massilia</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>6% <strong>Clavibacter</strong>.</td>
</tr>
<tr>
<td>NA</td>
<td>Conventional (n=8)</td>
<td>50% <strong>Bacillus</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>25% <strong>Staphylococcus</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>13% <strong>Paenibacillus</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>13% <strong>Lactobacillus</strong>.</td>
</tr>
<tr>
<td></td>
<td>Organic (n=11)</td>
<td>55% <strong>Bacillus</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>36% <strong>Enterobacter</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9% <strong>Ornithinibacillus</strong>.</td>
</tr>
</tbody>
</table>
Figure S 3-1 | Schematic illustration of the high-throughput method on 384 well plate

NC is negative control for one row consisting of only medium (either NB or PCB); PC is positive control for 3 rows consisting of only medium and isolate; A1-A6 are antibiotics one through six, with each antibiotic having two rows (low and high) consisting of medium and high concentrations of antibiotic.
References


Antibiotic resistance of faecal *Escherichia coli* in poultry, poultry farmers and poultry

Antibiotic resistance of faecal enterococci in poultry, poultry farmers and poultry


antimicrobial susceptibility in *Escherichia coli* isolated from pigs reared under

routes of administration of erythromycin in cattle. *J. Vet. Pharmacol. Therap.* **12**:289-
295.

multidrug-resistant bacteria isolated from a concentrated swine feeding operation.
*Environmental Health Perspectives* **113**, 137–142.

Cho, S., Fossler, C.P., Diez-Gonzales, F., Wells, S.J., Hedberg, C.W., Kaneene, J.B.,


Mathew, A. G., Beckmann, M.A., Saxton, A.M., A comparison of antibiotic resistance in bacteria isolated from swine herds in which antibiotics were used or excluded. *Journal of Swine Health and Production* **2001**, 9, 125-129.


*National Archives and Records Administration 2012a. Title 7: Agriculture, 2013, ecfr.gpoaccess.gov/cgi/t/text/text idx?c=ecfr&rgn=div5&view=text&node=7:3.1.1.9.32&idno=7#7:3.1.1.9.32.2.354.6*


USDA National Organic Program; National Archives and Records Administration 2012a. Title 7: Agriculture.


Chapter 4: Commercially available garden products as potential sources of antibiotic resistance genes—A survey

Introduction

Antimicrobial resistance stands to threaten the global public health response to infectious disease and is positioned to endanger “the very core of modern medicine.”\(^1,2\) Mediated at the genetic level, antibiotic resistance is predicated on the acquisition and expression of antibiotic resistance genes (ARGs), which are regarded as environmental contaminants of emerging concern,\(^3-5\) can be transferred among bacteria via horizontal gene transfer,\(^6\) and proliferate via selective and co-selective pressure.\(^7-9\)

With recent work verifying the role of environmental routes in pathogen emergence and disease transmission,\(^10-13\) it is critical to comprehensively consider environments existing at the nexus of human-ARG interaction. Understanding sources and scope of dissemination will prove critical in determining potential points of mitigation as well as approach.\(^11\)

Confined animal feeding operations (CAFOs) have widely been confirmed as sources of anthropogenic antimicrobial resistance into the environment.\(^14-16\) Conditions at CAFOs are sustained through regularly utilizing antibiotics at sub-therapeutic levels in animal feed as a method of infection treatment, prophylaxis, as well as growth promotion.\(^17\) An estimated 30-90% of antibiotics administered for agricultural use are not metabolized prior to excretion and are instead introduced into the environment where they exert a direct selective pressure for antimicrobial resistance.\(^18\) Additionally, consistent exposure to antibiotics drives selection for antibiotic resistant bacteria (ARB)
within the microbiome of CAFO animals, which are also excreted in manure and introduced to the environment.\textsuperscript{19}

Manure from CAFOs is frequently mixed with farmland soil to restore and replenish nutrients. This process constitutes a significant pathway of ARG dissemination from agriculture, with a substantial body of literature confirming that such repeated manure application results in accumulation of both antibiotics and ARGs in fields.\textsuperscript{15,20–25} However, there have been substantially fewer studies reporting on commercial manure products, which are readily obtainable by the general public, frequently branded as “organic,” and can reflect a more intimate human-ARG interaction than would living in the vicinity of manure amended farmland.

There is little transparency and regulation over garden product branding and treatment prior to purchase. At the time of writing, the U.S.A. code of regulations only specifies regulations of the term “organic” when used applied to food and fiber products, indicating that the term is not regulated when used in fertilizers.\textsuperscript{26} While nonprofit organizations such as the Organic Materials Review Institute (OMRI) have filled information voids through creating listings that extend U.S. National Organic Program standards to fertilizer input products, these input reviews do not consider propagation of antibiotic resistance, either through regulating antibiotic, ARB or ARG content.

In this study, we sought to survey and catalogue ARG loading in commercially available gardening products intended for home use as potting soil, garden soil, food and lawn fertilizer amendments, and manure amendments. Results were cross-referenced with package branding to determine if existing branding indicators may be correlated to ARG loading. Additionally, products were investigated for differences across manure sources
used and transparency of information on manure source and pretreatment prior to home application.

Antibiotic resistance genes sul1, ermF, tetL blaCTX, blatEM and vanA were quantified for 30 gardening products via qPCR. All products were analyzed with respect to per gram of gardening product and per 16srRNA copy, and correlation coefficients between individual ARGs and int1 copies were calculated.

**Materials and Methods**

*Soil and Amendment Selection*

Commercially-available garden products chosen for inclusion were classified into five major categories: Potting Soils, Garden Soils, Food Amendments, Lawn Amendments and Manure Products. Designations were based on product advertisement and intent of product use. Potting Soils were intended for initial planting of potted plants, with no need for an outside soil source. Garden Soils were defined as soil amendments used to supplement existing ground garden soil. Food amendments included fertilizer-based products intended to supplement a receiving soil with the objective of growing food for human consumption. Lawn amendments were fertilizer-based supplements intended to promote lawn growth in a receiving soil. Products in the manure category served the objective of restoring nutrients to soil and were advertised as strictly manure products. Manure products consisted of either chicken or steer manure. Gardening products specialized to specific plants (i.e. orchid, rose, etc…) were avoided when possible with preference given to products branded for general use.
All soil and amendment products included in this survey were commercially sold and readily obtainable in major garden and hardware stores via in-person purchase. Products were selected through conducting a survey of most frequently purchased items as advertised by stores commonly known to carry gardening products: Lowe’s, Home Depot, Walmart, Orchards and Anawalt, Lumber Co. Additional products were selected based on frequency of occurrence (i.e., products were consistently available across major hardware stores in Los Angeles, California). Advertised certifications were verified to the extent possible to ensure active certification as of May 2018.

**Sample Collection**

Soil and amendment bags were sampled for ARG quantification by measuring 0.25 ± .01g of each product into individual, sterile 2mL screw cap tubes preloaded with 1 ± 0.05 g 0.7mm garnet beads (Qiagen, Germantown, MD). All products were mixed by hand using sterile gloves, sampled in triplicate (n=90) and stored at -80 °C awaiting DNA extraction. After mixing, each product was loaded into individual 50 mL sterile Falcon tubes and stored at 4 °C awaiting loss on ignition testing. A quart of each product was additionally stored at -20 °C for hydrometer analysis.

**Soil and Amendment Characterization**

Moisture content for each commercial product was determined via dehydration at 105 °C over a period of 24 hours. Dehydration was verified as complete through confirming a less than 4% change in weight during a second measurement at the 25-hour mark. Loss on ignition was used to measure organic content in each product through
ignition at 550 °C over a period of two hours, as delineated by the U.S. Environmental Protection Agency (EPA). Particle size determination was accomplished through conducting hydrometer measurements.

**DNA Extraction**

All DNA extractions were completed using the DNeasy PowerSoil Kit (Qiagen, Germantown, MD) within two weeks of collection. Extractions proceeded per the manufacturer’s guidelines except for the cell lysis step, where a BioSpec MiniBeadbeater-8 (BioSpec Products, Bartlesville, OK) was used in place of vortexing for a duration of 2 mins. Every extraction included an additional 2 mL screw cap tube preloaded with 1 ± 0.05 g of 0.7mm diameter garnet beads (Qiagen, Germantown, MD) absent of sample as an extraction blank to confirm absence of contamination during the extraction process. Eluted DNA was aliquoted and stored at -20 °C awaiting qPCR analysis. Total DNA concentration was measured using UV absorption via a Nanodrop 2000C (Thermo Scientific, Waltham MA), as were 260/280 absorbance ratios.

**qPCR**

All products were analyzed for ARG abundance of *sul1, ermF, tetL, blaCTX*, *blaTEM* and *vanA, int1* and the 16S-rRNA gene, (a total bacteria surrogate measure) via qPCR. All assays were conducted using PowerUp SYBR Green Master Mix and consisted of a 25 μL reaction volume: 12.5 μl of PowerUp SYBR Green MasterMix (Life Technologies, Grand Island, NY), 1.25 μL of each primer, forward and reverse, 2 μL of template DNA, and 8 μL of molecular grade. Primers and primer concentrations can be
found in Table 4-1 and were used as developed and validated previously in the literature.\textsuperscript{30–35} Each assay run included a 7-point standard curve positive control, applicable extraction blanks and a negative control of molecular grade water, with each sample plated in triplicate wells. All assays were performed in 96-well reaction plates using StepOne Plus (Applied Biosystems). Temperature cycles used consisted of 95°C (10 mins) followed by 40 cycles of 95°C (10 sec), gene specific annealing and elongation settings and finally, a melt curve stage consisting of 95°C for 1 minute followed by a ramp increase from 50-95°C. Annealing and elongation settings can be found in Table 4-1 and were as reported previously in literature.\textsuperscript{30–35}

All sample DNA extracts were diluted to a concentration of 1.0 ng/µL to correct for inhibition effects during qPCR. Well spike and serial dilution tests were used to determine the appropriate DNA concentration and dilutions were accomplished using molecular grade water. Serial dilution involved picking random samples and serially diluting DNA by a factor of 4 and performing qPCR on all dilutions to determine the ideal dilution factor. All study samples were quantified for DNA concentration and the dilution factor was used in conjunction with the lowest DNA yield to determine an appropriate standardized dilution concentration. A second set of randomly selected samples were then used to verify absence of inhibition through performing well spikes. Unquantifiable samples were run at higher concentrations to verify absence of gene and ensure that over-dilution was not occurring. Dilution factors were back calculated during data analysis to reverse this effect and obtain per mass quantification.

Standard curves were designed using sequences obtained through the NCBI database and ordered through IDT Technologies.\textsuperscript{36} Known concentrations of the designed
DNA fragment were run alongside environmental samples, yielding a seven-point standard curve and allowing for quantitation of gene copies. Standard curve efficiencies were 100 ± 6% across all qPCR assays and all R² values were at or above 0.99. Melt curves were used to further verify target gene amplification specificity.

**Results and Discussion**

**Soil and Amendment Selection**

A total of 10 potting soils, 7 garden soils, 5 food amendments, 4 lawn amendments and 4 manure products were characterized and quantified for antibiotic resistance *sul1, ermF, tetL, blaCTX, blaTEM* and *vanA* as well as 16S rRNA and *int1*. ARGs were selected to represent a range of genes proposed as possible indicators of the resistance status of environmental settings.³⁷ Gardening products selected for survey inclusion spanned 16 brands and reported manure sources ranging from poultry litter and dairy cow manure to bat guano. Product names, manufacturer, manure source, branding information and certifications are summarized in Table 4-2.

Overall “Natural” and “Organic” were the most common branding terms associated with products samples and poultry litter was the most commonly used manure source, with 10 products failing to list the manure type used in their products. 13 products claimed either Demeter or OMRI certification. A summary of certifications can be found in Table 4-2.
ARG quantities in garden products

*ermF, sul1, tetL* and *int1* genes were consistently quantified in products included in this study with no products found to contain the genes *bla_{CTX}, bla_{TEM*} or *vanA*. While a standard control was performed for the *bla_{CTX}, bla_{TEM*} and *vanA* assays, environmental samples of confirmed gene content will be applied to confirm absence.

*ermF, sul1* and *tetL* confer resistance to macrolides sulfonamides, and tetracyclines respectively, which have been reported as approved for use in food-producing animals within the United States as recently as 2012. Macrolides made up a reported 4.5% (616.27 tons) of total antimicrobial use in 2012 while sulfonamides made up an estimated 3.6% (493.51 tons). Tetracyclines made up 43.9% (5,954.36 tons) of total antimicrobial use in 2012, the largest of any antibiotic class. 38

Among the genes not quantifiable, *vanA* confers resistance to vancomycin, which is currently not approved for agricultural use, and *bla_{CTX}, bla_{TEM*} confer resistance to penicillins, which are approved for use in food producing animals and constituted an estimated 965.2 tons of the 13,569.04 tons of total antimicrobial use in the United States in 2012 (approximately 7.11%). 38

*ermF*

*ermF* was detected in six out of the ten potting soils surveyed, with detection in only one of three sampling triplicates for B1. B3, B6, B8, B9 and B10 were all consistently detectable for *ermF*, with quantities ranging from $4.88 \times 10^3$ to $1.09 \times 10^6$ *ermF* gene copies per gram of soil (Table 4-4), and from $2.47 \times 10^5$ to $4.18 \times 10^3$ *ermF* gene copies/16s rRNA gene copies (Table 4-5). Of the consistently detected samples, B9
and B10 reported the lowest quantities of the \textit{erm}F gene when analyzed relative to mass of soil and relative to 16s rRNA gene copies. B3 and B6 consistently reported a higher \textit{erm}F content regardless of analytical approach.

\textit{erm}F was consistently detected in all garden soils selected for analysis with the exception of B14. \textit{erm}F quantities in garden soil spanned from a minimum of $1.36 \times 10^4$ \textit{erm}F gene copies per gram of soil to a maximum of $6.79 \times 10^5$ \textit{erm}F gene copies per gram of soil (Table 4-4). When analyzed by 16s rRNA gene copy, \textit{erm}F relative copies ranged from $1.48 \times 10^4$ to $1.94 \times 10^3$ \textit{erm}F gene copies/16s rRNA gene copies (Table 4-5).

\textit{erm}F was consistently quantified in four out of the five food amendment products included in this survey, with B23 found to be the only product to not contain a quantifiable amount of \textit{erm}F copies. Of the quantifiable samples, copy numbers per gram of product ranged from $3.56 \times 10^4$ to $7.69 \times 10^5$ \textit{erm}F gene copies per gram of soil. Relative to 16s rRNA, \textit{erm}F relative abundances ranged from $1.11 \times 10^3$ to $1.24 \times 10^2$ \textit{erm}F/16s rRNA (Table 4-4 and Table 4-5).

The \textit{erm}F gene was consistently detected across all lawn amendment and manure products included in this survey and ranged from $4.42 \times 10^2$ to $1.21 \times 10^6$, and $1.43 \times 10^5$ to $1.38 \times 10^7$ \textit{erm}F copies per gram of product, respectively. Copy numbers relative to 16s rRNA copies ranged from $4.17 \times 10^{-4}$ to $6.22 \times 10^{-2}$, and $1.51 \times 10^3$ to $8.31 \times 10^2$ \textit{erm}F gene copies/16s rRNA gene copies, respectively (Table 4-4 and Table 4-5).

\textit{sul}1

\textit{sul}1 was consistently quantifiable in eight out of ten potting soil products selected for survey inclusion and ranged from $1.7 \times 10^2$ to $5.91 \times 10^5$ \textit{erm}F gene copies per gram.
of product (See Table 4-4 and Table 4-5). Relative to 16s rRNA gene copy number, potting soils samples were found to have a sul1/16s rRNA ratio of ranging from $1.32 \times 10^{-4}$ to $8.25 \times 10^{3}$ ermA gene copies/16s rRNA gene copies.

Consistent sul1 quantification was observed for all other product categories and ranged from $1.7 \times 10^{2} - 5.91 \times 10^{5}$, $2.14 \times 10^{4} - 2.78 \times 10^{6}$, $3.41 \times 10^{3} - 9.89 \times 10^{5}$, $1.92 \times 10^{2} - 2.28 \times 10^{6}$, and $1.38 \times 10^{5} - 1.11 \times 10^{6}$ copies per gram of product for garden soil, food amendment, lawn amendment and manure samples, respectively (Table 4-4). Manure products proved to show the least variability between products, with the range of copies per gram of product spanning less than one order of magnitude.

When analyzing by copies relative to 16s rRNA copies, garden soils ranged from $1.32 \times 10^{-4} - 8.25 \times 10^{3}$ sul1/16s rRNA, food amendments ranged from $2.46 \times 10^{-4}$ to $1.44 \times 10^{2}$ sul1/16s rRNA, lawn amendments ranged from $6.03 \times 10^{-4}$ to $2.72 \times 10^{-2}$ sul1/16s rRNA and manure amendments ranged from $1.69 \times 10^{3}$ to $8.67 \times 10^{3}$ sul1/16s rRNA. Consistent with results per gram of product, manure samples were the least variable among products, varying by less than one order of magnitude (Table 4-5).

**tetL**

Potting soil samples proved to be highly variable in tetL gene presence across brands, with only three of ten products consistently reporting gene presence. Two additional products confirmed tetL presence in two out of three sampling triplicates and three additional products confirmed presence in only one out of three sampling triplicates. Two samples failed to confirm tetL presence in any of the triplicates measured. Of the samples showing consistent gene presence, values ranged from $8.53 \times 10^{-4}$ to
10² – 8.81 x 10⁴ tetL gene copies per gram of soil. When normalized by 16s rRNA gene copies, values ranged from 6.23 x 10⁻⁶ – 5.63 x 10⁻⁵ tetL gene copies/16s rRNA gene copies.

tetL was consistently quantifiable in four out of seven garden soil products, with the remaining three products amplifying for only one of three sampling replicates each. Of the consistently quantifiable products, tetL gene copies per gram of soil ranged from 2.21 x 10³ to 4.91 x 10⁵. When analyzed with respect to 16s rRNA gene copy number values ranged from 9.65 x 10⁻⁶ – 7.51 x 10⁻⁴ tetL gene copies/16s rRNA gene copies.

Food amendment, lawn amendment and manure samples were all found to contain a consistently quantifiable tetL gene content. Quantities ranged from 7.61 x 10²-1.70 x 10⁷ tetL gene copies per gram of product for food amendments, 2.64 x 10³-5.67 x 10⁴ tetL gene copies per gram of product for lawn amendments and 1.09 x 10³-1.18 x 10⁶ tetL gene copies per gram of product for manure garden products. Quantities with respect to 16s rRNA gene copy number ranged from 3.54 x 10⁻⁵-4.14 x 10⁻¹ tetL gene copies/16s rRNA gene copies for food amendments, 1.10 x 10⁻⁵-8.07 x 10⁻⁴ tetL gene copies/16s rRNA gene copies for lawn amendments and 1.15 x 10⁻⁵-1.36 x 10⁻² tetL gene copies/16s rRNA gene copies for manure garden products.

int1

Presence of int1 gene was verified and quantified for all but two of the potting soil samples included in this study (B2 and B7). int1 quantities ranged from 2.52 x 10² to 9.56 x 10⁵ for quantifiable potting soil products, a range spanning 3.57 orders of magnitude. Garden soil, food amendment, lawn amendment and manure products all
consistently confirmed presence of \textit{int1} and reported ranges of $1.25 \times 10^4$-$2.58 \times 10^6$, $6 \times 10^3$ to $1.5 \times 10^6$, $6.39 \times 10^2$ to $1.81 \times 10^6$ and $4.79 \times 10^4$ to $9.88 \times 10^5$ gene copies per gram of soil respectively. Ranges spanned 2.3, 2.4, 3.5 and 1.3 orders of magnitude for garden soil, food amendment, lawn amendment and manure products, respectively (Table 4-4).

When normalized by 16s rRNA gene copy, quantifiable potting soil products ranged from $5.13 \times 10^{-6}$ to $3.7 \times 10^{-3}$, 2.85 orders of magnitude. Garden soil \textit{int1} gene copies over 16s rRNA gene copies ranged from $7.74 \times 10^{-5}$ to $8.25 \times 10^{-3}$, a difference of 2.03 orders of magnitude. Food amendments and lawn amendments had similar distributions, ranging from $1.49 \times 10^{-4}$ to $2.25 \times 10^{-2}$ \textit{int1}/16s rRNA (2.22 orders of magnitude) and $2.78 \times 10^{-4}$ to $6.85 \times 10^{-2}$ \textit{int1}/16s rRNA (2.39 orders of magnitude), respectively. Manure samples ranged from $7.02 \times 10^{-4}$ to $7.32 \times 10^{-3}$ \textit{int1}/16s rRNA, spanning 1.02 orders of magnitude (Table 4-5).

\textbf{Inter-gene observations}

\textit{int1} and \textit{sul1} correlations

\textit{int1} and \textit{sul1} data further confirm results of several environmental studies finding strong associations between \textit{sul1} and \textit{int1} gene quantities.\textsuperscript{39,40} In the present study, \textit{int1} is found to exhibit a strong positive correlation with \textit{sul1} for both methods of analysis. The Pearson correlation coefficient calculated on a per gram of product basis was determined to be $r = 0.8722$ ($R^2=.7607$, $p < 0.01$) for \textit{sul1} vs. \textit{int1} and only $r = 0.2601$ ($R^2=.0677$, $p < 0.05$) for \textit{ermF} vs. \textit{int1}.
Correlations proved to be more strongly positive when analyzing per 16s rRNA gene copy, where \( int \) and \( sul \) correlation yielded a Pearson coefficient of \( r = 0.8968 \) (\( R^2 = 0.8043, p < 0.01 \)), contrasted by only a moderate positive correlation between \( ermF \) and \( int \) (\( r=0.5697, R^2=0.3256, p < 0.01 \)).

\textit{ARG frequency of detection}

\( ermF \) and \( tetL \) were found to be less abundant overall when compared to \( sul \), with products unquantifiable for \( sul \) consistently unquantifiable for \( ermF \), and only one sample not found to contain \( sul \) confirming presence for \( tetL \). This is also consistent with environmental data, which shows \( sul \) to be significantly more abundant with regards to presence-absence.\(^{36}\) Interestingly, while environmental data show differences between \( sul \) and \( ermF \) to span several orders of magnitude, data presented here often vary between genes at approximately one order of magnitude, with \( ermF \) quantities occasionally surpassing those of \( sul \).

\textit{ARG quantities in environmental context}

When compared to ARG quantities reported regionally throughout California, U.S.A., \( sul \) quantities are comparable or below values reported in public parks.\(^{36}\) Gene copy numbers per gram of soil in public parks generally fluctuated over two orders of magnitude, between \( 1 \times 10^5 \) - \( 1 \times 10^7 \), while garden products were found to largely fluctuate between \( 1 \times 10^4 \) and \( 1 \times 10^6 \). In contrast, while environmental quantities of \( ermF \) fluctuated between 10 and 1000 gene copies per gram of soil in public parks, quantities reported here consistently fall between \( 1 \times 10^4 \) and \( 1 \times 10^6 \) gene copies per gram of
product, suggesting that addition of gardening products may introduce a greater overall shift in *erm*F gene quantities than in *sul1* quantities when applied to background soils.

**Third party certifications and ARGs**

OMRI was the most commonly advertised third party certification method, with 37% of products included in this survey listed as OMRI approved (Figure 4-1). OMRI listed products were found to contain gene quantities comparable to non-listed products, indicating that despite verifying that input products are compliant with organic standards, OMRI certification cannot serve as an indicator for ARG introduction via garden supplies. Further, this implies that the extension of organic standards, as defined in the United States by the Code of Federal Regulations, currently applied to food and fiber products may prove insufficient in mitigating ARG introduction via gardening supplies.

Demeter certification applies exclusively to biodynamic farms and corresponded to one brand included in this survey, Malibu Compost (B9 and B23). While the compost product proved to be the only food amendment product unquantifiable for *erm*F, the potting soil sold by the same brand and advertising the same certification was quantifiable for *erm*F in addition to *sul1* and int1. More products of this certification are needed to determine if Demeter certification may serve as an ARG predictor.

**ARGs and manure sourcing**

Manure source was not consistently available across gardening products. Upon surveying technical assistance resources, even when manure source was available, proportions of manure used in product are considered proprietary, as were pretreatments
of manure, indicating that there exists a large dearth of information available to consumers. This largely limits the ability to screen for pre-treatment effects on final product ARG levels. Clear and transparent information concerning manure source and treatment will prove a clear and essential first step in understanding mitigation strategies for ARG input via gardening products.
<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer</th>
<th>Concentration</th>
<th>Sequence (5’-3’)</th>
<th>Annealing Conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>16S-rRNA-F</td>
<td>(100) nM</td>
<td>CCTACGGGAGGCAGCAG</td>
<td>60 °C/60 s</td>
<td>(31)</td>
</tr>
<tr>
<td></td>
<td>16S-rRNA-R</td>
<td>(100) nM</td>
<td>ATTACCGCGGCTGCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>sul</em>1</td>
<td><em>sul</em>1-F</td>
<td>(200) nM</td>
<td>CGCACCAGGAACATGCACAG</td>
<td>65 °C/60 s</td>
<td>(33)</td>
</tr>
<tr>
<td></td>
<td><em>sul</em>1-R</td>
<td>(200) nM</td>
<td>TGAAGTCCGCGAGAGGCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>bla</em>CTX</td>
<td><em>bla</em>CTX-F</td>
<td>(500) nM</td>
<td>CTATGCGAACCACACGATA</td>
<td>60 °C/30 s 72 °C/15 s</td>
<td>(32)</td>
</tr>
<tr>
<td></td>
<td><em>bla</em>CTX-R</td>
<td>(500) nM</td>
<td>ACGGCTTTCTGCTTAGGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>van</em>A</td>
<td><em>van</em>A-F</td>
<td>(300) nM</td>
<td>CTGTGAGGTCGGTGTCG</td>
<td>60 °C/60 s</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td><em>van</em>A-R</td>
<td>(300) nM</td>
<td>TTTGGTCCACCTGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>erm</em>F</td>
<td><em>erm</em>F-F</td>
<td>(500) nM</td>
<td>TCGTTTACGGGTCACTT</td>
<td>60 °C/30 s</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td><em>erm</em>F-R</td>
<td>(500) nM</td>
<td>CAACCAAGCTGCTGTGTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>bla</em>TEM</td>
<td><em>bla</em>TEM-F</td>
<td>(400) nM</td>
<td>TGGGGGAAATGTGC</td>
<td>50 °C/60 s 72 °C/60 s</td>
<td>(30)</td>
</tr>
<tr>
<td></td>
<td><em>bla</em>TEM-R</td>
<td>(400) nM</td>
<td>GGAATAAGGGGCGACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tet</em>L</td>
<td><em>tet</em>L-F</td>
<td>(900) nM</td>
<td>GGTGTTAAGCTCTACCTGAT</td>
<td>60 °C/30 s</td>
<td>(35)</td>
</tr>
<tr>
<td></td>
<td><em>tet</em>L-R</td>
<td>(900) nM</td>
<td>CCAATGGAAAGGTTAATATAAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>int1</td>
<td>int1-F</td>
<td>(200) nM</td>
<td>GGCTTCGTGATCCTGCTT</td>
<td>55 °C/30 s 72 °C/30 s</td>
<td>(34)</td>
</tr>
<tr>
<td></td>
<td>int1-R</td>
<td>(200) nM</td>
<td>CATTCCCCTGGCGGTGGTTT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Table 4-2 | Garden product sample identification

<table>
<thead>
<tr>
<th>Product Type</th>
<th>Sample ID</th>
<th>Product Name</th>
<th>Brand Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Potting Soil</strong></td>
<td>B1</td>
<td>Potting Mix</td>
<td>Miracle Gro</td>
</tr>
<tr>
<td></td>
<td>B2</td>
<td>Edna’s best potting soil</td>
<td>EB Stone</td>
</tr>
<tr>
<td></td>
<td>B3</td>
<td>Patio Plus Premium Outdoor Potting Mix</td>
<td>Kellogg</td>
</tr>
<tr>
<td></td>
<td>B4</td>
<td>Moisture Control Potting Mix</td>
<td>Miracle Gro</td>
</tr>
<tr>
<td></td>
<td>B5</td>
<td>Natural and Organic Potting Mix</td>
<td>Jobe’s Organics</td>
</tr>
<tr>
<td></td>
<td>B6</td>
<td>Black Gold All Purpose Potting Mix</td>
<td>Sungro</td>
</tr>
<tr>
<td></td>
<td>B7</td>
<td>Organic Potting Mix for all potted plants</td>
<td>Espoma</td>
</tr>
<tr>
<td></td>
<td>B8</td>
<td>All Purpose Potting Mix</td>
<td>Vigoro</td>
</tr>
<tr>
<td></td>
<td>B9</td>
<td>Baby Bu’s Biodynamic Blend Potting Soil</td>
<td>Malibl Compost</td>
</tr>
<tr>
<td></td>
<td>B10</td>
<td>Natural + Organic Potting Mix</td>
<td>Ecoscraps</td>
</tr>
<tr>
<td><strong>Garden Soil</strong></td>
<td>B11</td>
<td>All Natural Garden Soil</td>
<td>Kellogg</td>
</tr>
<tr>
<td></td>
<td>B12</td>
<td>Organic Gardening Soil</td>
<td>Nature’s Care</td>
</tr>
<tr>
<td></td>
<td>B13</td>
<td>Premium All Purpose Planting Mix</td>
<td>Dr Earth</td>
</tr>
<tr>
<td></td>
<td>B14</td>
<td>Organic Garden Soil</td>
<td>Ecoscraps</td>
</tr>
<tr>
<td></td>
<td>B15</td>
<td>Flower &amp; Vegetable Garden Soil</td>
<td>Sta Green</td>
</tr>
<tr>
<td></td>
<td>B16</td>
<td>All Purpose Garden Soil</td>
<td>Vigoro</td>
</tr>
<tr>
<td></td>
<td>B17</td>
<td>All Purpose Garden Soil</td>
<td>Miracle Gro</td>
</tr>
<tr>
<td></td>
<td>B21</td>
<td>Organic Plus: Tomato, Vegetable</td>
<td>Kellogg</td>
</tr>
<tr>
<td></td>
<td>B22</td>
<td>Garden-tone: Herb and Vegetable Food</td>
<td>Espoma</td>
</tr>
<tr>
<td></td>
<td>B23</td>
<td>Bu’s Blend Biodynamic Compost</td>
<td>Malibl Compost</td>
</tr>
<tr>
<td></td>
<td>B24</td>
<td>Vegetable and Tomato</td>
<td>Jobe’s Organics</td>
</tr>
<tr>
<td></td>
<td>B25</td>
<td>Starter and Transplanting Granular Plant Food</td>
<td>Burpee</td>
</tr>
<tr>
<td><strong>Fruit Amendment</strong></td>
<td>B31</td>
<td>Organic Plus: Topper Soil for Lawns, Sod, and Seed</td>
<td>Kellogg</td>
</tr>
<tr>
<td></td>
<td>B32</td>
<td>Tree &amp; Shrub Garden Soil Plus Fertilizer</td>
<td>Sta Green</td>
</tr>
<tr>
<td></td>
<td>B33</td>
<td>Turf Max</td>
<td>Green As It Gets</td>
</tr>
<tr>
<td></td>
<td>B34</td>
<td>Palm, Cactus, and Citrus</td>
<td>Kellogg</td>
</tr>
<tr>
<td><strong>Lawn Amendment</strong></td>
<td>B41</td>
<td>Steer manure Blend</td>
<td>Earthgro</td>
</tr>
<tr>
<td></td>
<td>B42</td>
<td>Steer Manure</td>
<td>Gardeners</td>
</tr>
<tr>
<td></td>
<td>B51</td>
<td>Composted Chicken Manure</td>
<td>G&amp;B Organics</td>
</tr>
<tr>
<td></td>
<td>B52</td>
<td>Chicken Manure</td>
<td>Earthgro</td>
</tr>
</tbody>
</table>
### Table 4-3 | Garden Product Characteristics

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Certifications</th>
<th>Branding</th>
<th>Manure Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OMRI</td>
<td>Demeter</td>
<td>Organic</td>
</tr>
<tr>
<td>B1</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B2</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B3</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B4</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B5</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B7</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B8</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B9</td>
<td>No</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>B10</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B11</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B12</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B13</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B14</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B15</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B16</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B17</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B21</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B22</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B23</td>
<td>No</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>B24</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B25</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B31</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B32</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B33</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B34</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B41</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B42</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B51</td>
<td>Yes</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>B52</td>
<td>No</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ermF</td>
<td>tetL</td>
<td>sul1</td>
</tr>
<tr>
<td>----------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td><strong>Potting Soil</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>3.2E+02</td>
<td>4.6E+03</td>
<td>1.3E+04</td>
</tr>
<tr>
<td>B2</td>
<td>0.0E+00</td>
<td>0.0E+00</td>
<td>6.0E+02</td>
</tr>
<tr>
<td>B3</td>
<td>9.2E+05</td>
<td>6.8E+04</td>
<td>2.0E+05</td>
</tr>
<tr>
<td>B4</td>
<td>0.0E+00</td>
<td>2.3E+02</td>
<td>1.1E+04</td>
</tr>
<tr>
<td>B5</td>
<td>0.0E+00</td>
<td>4.8E+02</td>
<td>4.8E+02</td>
</tr>
<tr>
<td>B6</td>
<td>3.0E+05</td>
<td>0.0E+00</td>
<td>1.2E+04</td>
</tr>
<tr>
<td>B7</td>
<td>0.0E+00</td>
<td>1.1E+03</td>
<td>0.0E+00</td>
</tr>
<tr>
<td>B8</td>
<td>2.7E+05</td>
<td>7.2E+02</td>
<td>5.6E+05</td>
</tr>
<tr>
<td>B9</td>
<td>8.5E+03</td>
<td>3.0E+03</td>
<td>9.2E+03</td>
</tr>
<tr>
<td>B10</td>
<td>1.4E+04</td>
<td>8.9E+02</td>
<td>1.4E+05</td>
</tr>
<tr>
<td><strong>Garden Soil</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B11</td>
<td>1.4E+05</td>
<td>2.5E+05</td>
<td>8.9E+05</td>
</tr>
<tr>
<td>B12</td>
<td>1.8E+05</td>
<td>2.6E+04</td>
<td>5.0E+05</td>
</tr>
<tr>
<td>B13</td>
<td>4.4E+04</td>
<td>9.6E+03</td>
<td>1.6E+05</td>
</tr>
<tr>
<td>B14</td>
<td>0.0E+00</td>
<td>2.9E+02</td>
<td>2.6E+04</td>
</tr>
<tr>
<td>B15</td>
<td>1.7E+05</td>
<td>2.6E+02</td>
<td>3.3E+05</td>
</tr>
<tr>
<td>B16</td>
<td>5.9E+05</td>
<td>5.0E+03</td>
<td>2.4E+06</td>
</tr>
<tr>
<td>B17</td>
<td>1.4E+05</td>
<td>4.6E+02</td>
<td>3.4E+05</td>
</tr>
<tr>
<td><strong>Food Amendment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B21</td>
<td>6.5E+05</td>
<td>9.0E+06</td>
<td>9.2E+05</td>
</tr>
<tr>
<td>B22</td>
<td>8.2E+04</td>
<td>1.1E+07</td>
<td>4.1E+05</td>
</tr>
<tr>
<td>B23</td>
<td>0.0E+00</td>
<td>9.9E+03</td>
<td>6.5E+04</td>
</tr>
<tr>
<td>B24</td>
<td>5.5E+04</td>
<td>9.8E+04</td>
<td>3.2E+04</td>
</tr>
<tr>
<td>B25</td>
<td>4.8E+05</td>
<td>8.3E+05</td>
<td>4.8E+05</td>
</tr>
<tr>
<td><strong>Lawn Amendment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B31</td>
<td>1.1E+05</td>
<td>1.9E+04</td>
<td>2.0E+06</td>
</tr>
<tr>
<td>B32</td>
<td>4.2E+05</td>
<td>2.1E+03</td>
<td>1.2E+05</td>
</tr>
<tr>
<td>B33</td>
<td>7.1E+05</td>
<td>5.8E+03</td>
<td>4.0E+05</td>
</tr>
<tr>
<td>B34</td>
<td>2.8E+05</td>
<td>4.1E+04</td>
<td>4.7E+04</td>
</tr>
<tr>
<td><strong>Manure</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B41</td>
<td>2.4E+05</td>
<td>2.6E+03</td>
<td>1.7E+05</td>
</tr>
<tr>
<td>B42</td>
<td>4.8E+05</td>
<td>9.5E+04</td>
<td>1.7E+05</td>
</tr>
<tr>
<td>B51</td>
<td>7.0E+06</td>
<td>1.6E+04</td>
<td>1.0E+06</td>
</tr>
<tr>
<td>B52</td>
<td>2.3E+06</td>
<td>9.4E+05</td>
<td>5.3E+05</td>
</tr>
</tbody>
</table>
Table 4-5 | ARG copy gene copies per 16s-rRNA gene copies

<table>
<thead>
<tr>
<th></th>
<th>ermF</th>
<th>tetL</th>
<th>sul1</th>
<th>int1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Potting Soil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>9.7E-06</td>
<td>4.2E-05</td>
<td>1.2E-04</td>
<td>8.0E-05</td>
</tr>
<tr>
<td>B2</td>
<td>0.0E+00</td>
<td>0.0E+00</td>
<td>7.5E-06</td>
<td>0.0E+00</td>
</tr>
<tr>
<td>B3</td>
<td>3.3E-03</td>
<td>2.5E-04</td>
<td>7.3E-04</td>
<td>9.7E-04</td>
</tr>
<tr>
<td>B4</td>
<td>0.0E+00</td>
<td>1.2E-06</td>
<td>1.5E-04</td>
<td>4.4E-05</td>
</tr>
<tr>
<td>B5</td>
<td>0.0E+00</td>
<td>9.2E-06</td>
<td>9.1E-06</td>
<td>3.7E-06</td>
</tr>
<tr>
<td>B6</td>
<td>2.4E-03</td>
<td>0.0E+00</td>
<td>9.9E-05</td>
<td>3.4E-05</td>
</tr>
<tr>
<td>B7</td>
<td>0.0E+00</td>
<td>2.0E-05</td>
<td>0.0E+00</td>
<td>0.0E+00</td>
</tr>
<tr>
<td>B8</td>
<td>1.1E-03</td>
<td>2.8E-06</td>
<td>2.2E-03</td>
<td>3.2E-03</td>
</tr>
<tr>
<td>B9</td>
<td>6.0E-05</td>
<td>2.0E-05</td>
<td>6.7E-05</td>
<td>4.1E-05</td>
</tr>
<tr>
<td>B10</td>
<td>3.9E-05</td>
<td>1.6E-06</td>
<td>3.7E-04</td>
<td>1.7E-04</td>
</tr>
<tr>
<td><strong>Garden Soil</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B11</td>
<td>4.5E-04</td>
<td>6.2E-04</td>
<td>2.5E-03</td>
<td>8.1E-04</td>
</tr>
<tr>
<td>B12</td>
<td>5.3E-04</td>
<td>6.4E-05</td>
<td>1.7E-03</td>
<td>3.8E-04</td>
</tr>
<tr>
<td>B13</td>
<td>3.4E-04</td>
<td>7.0E-05</td>
<td>1.2E-03</td>
<td>2.5E-04</td>
</tr>
<tr>
<td>B14</td>
<td>0.0E+00</td>
<td>1.8E-06</td>
<td>1.5E-04</td>
<td>1.1E-04</td>
</tr>
<tr>
<td>B15</td>
<td>7.7E-04</td>
<td>9.4E-07</td>
<td>1.4E-03</td>
<td>3.2E-04</td>
</tr>
<tr>
<td>B16</td>
<td>1.6E-03</td>
<td>1.4E-05</td>
<td>6.5E-03</td>
<td>6.3E-03</td>
</tr>
<tr>
<td>B17</td>
<td>2.6E-04</td>
<td>7.3E-07</td>
<td>6.3E-04</td>
<td>2.9E-04</td>
</tr>
<tr>
<td><strong>Food Amendment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B21</td>
<td>1.2E-03</td>
<td>1.7E-02</td>
<td>1.7E-03</td>
<td>2.3E-03</td>
</tr>
<tr>
<td>B22</td>
<td>3.0E-03</td>
<td>3.5E-01</td>
<td>1.3E-02</td>
<td>1.5E-02</td>
</tr>
<tr>
<td>B23</td>
<td>0.0E+00</td>
<td>5.7E-05</td>
<td>3.7E-04</td>
<td>3.8E-04</td>
</tr>
<tr>
<td>B24</td>
<td>1.0E-02</td>
<td>1.9E-02</td>
<td>4.2E-03</td>
<td>9.8E-03</td>
</tr>
<tr>
<td>B25</td>
<td>4.0E-03</td>
<td>6.8E-03</td>
<td>3.9E-03</td>
<td>7.3E-03</td>
</tr>
<tr>
<td><strong>Lawn Amendment</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B31</td>
<td>4.8E-04</td>
<td>8.1E-05</td>
<td>8.5E-03</td>
<td>4.1E-03</td>
</tr>
<tr>
<td>B32</td>
<td>3.0E-03</td>
<td>1.5E-05</td>
<td>9.5E-04</td>
<td>3.2E-04</td>
</tr>
<tr>
<td>B33</td>
<td>4.9E-02</td>
<td>2.4E-04</td>
<td>2.3E-02</td>
<td>5.3E-02</td>
</tr>
<tr>
<td>B34</td>
<td>4.3E-03</td>
<td>6.2E-04</td>
<td>7.3E-04</td>
<td>1.5E-03</td>
</tr>
<tr>
<td><strong>Manure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B41</td>
<td>2.5E-03</td>
<td>2.8E-05</td>
<td>1.8E-03</td>
<td>1.1E-03</td>
</tr>
<tr>
<td>B42</td>
<td>6.4E-03</td>
<td>1.3E-03</td>
<td>2.2E-03</td>
<td>9.3E-04</td>
</tr>
<tr>
<td>B51</td>
<td>4.4E-02</td>
<td>1.1E-04</td>
<td>6.9E-03</td>
<td>6.1E-03</td>
</tr>
<tr>
<td>B52</td>
<td>2.8E-02</td>
<td>1.2E-02</td>
<td>7.1E-03</td>
<td>7.1E-03</td>
</tr>
</tbody>
</table>
Figure 4-1 | OMRI certified to non-certified products

OMRI certified garden products are filled in orange and non-certified can be identified by a blue fill color.
References

(1) Organization, W. H. Global action plan on antimicrobial resistance. 2015.


(25) Marti, R.; Scott, A.; Tien, Y. C.; Murray, R.; Sabourin, L.; Zhang, Y.; Topp, E. Impact of manure fertilization on the abundance of antibiotic-resistant bacteria and frequency of


(38) Economou, V.; Gousia, P. Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infect. Drug Resist.* 2015, 8, 49–61.


Chapter 5: Environmental Fate of Antibiotic Resistance Genes Introduced through Application of Commercially Available Garden Products

Introduction

The consequences of antibiotic resistance stands are already felt globally, with a minimum number of deaths due to antibiotic resistance each year estimated at 700,000.\(^1,2\) Antibiotic resistance is mediated genetically through expression of antibiotic resistance genes (ARGs) which have been confirmed to be both ubiquitous in the environment and transferrable via horizontal gene transfer. Considered contaminants of emerging concern, once in the environment ARGs quantities may persist, decay or proliferate, making them unique contaminants.

Environmental ARG quantities have been shown to be rising due to anthropogenic influence, with the environment playing an important role as an ARG reservoir and site for pathogen emergence.\(^3\)–\(^6\) Human activities linked to environmental ARG inputs include processes resulting from human antibiotic use such as reclaimed water use\(^7\)–\(^9\) and biosolids\(^10\) application, as well as antibiotics used to sustain agricultural practices, as is the case in confined animal feeding operations.\(^11\)–\(^13\)

Manure generated through confined animal feeding operations (CAFOs) constitutes and anthropogenic ARG source into the environment due to antibiotic use in animal feed, with antibiotics used as a method for prophylaxis and growth promotion.\(^11\)–\(^15\) Application of CAFO-derived manure, containing antibiotics as well as antibiotic resistant bacteria and antibiotic resistance genes, to agricultural soil has been confirmed
as an anthropogenic ARG source, with a wide collection of work has revealing accumulation of antibiotic resistance genes upon repeated application.\textsuperscript{16,12,17–21}

While many studies have confirmed accumulation through monitoring field scale application, fate and transport studies are uncommon. Of the literature that exists, discrepancies regarding decay timelines vary widely from mere days to months, suggesting that a great need for additional work to aid in identifying circumstances prompting ARG proliferation and decay.\textsuperscript{18,20,22–25}

Additionally, while some studies have aimed to understand the fate and transport of ARGs sourced from manure used in large scale agricultural settings, to our knowledge this is the first study to explore fate and transport of ARGs derived from commercially available products, which may signal a more intimate route of human-ARG interaction.

In this study, we sought to understand the behavior of ARGs sourced from garden products upon application to a receiving soil. Through constructing microcosms mimicking the input of garden products verified for ARG content into a receiving ARG-free soil, we monitored changes in ARG copy numbers over time. Temporal profiles were plotted to observe differences in proliferation/decay between garden products and genes. Additionally, by measuring soil at the surface as well as 1.5in deep, we sought to explore whether solar degradation may play a role at soil depths relevant to public health.

**Materials and Methods**

**Soil and Treatment Selection**

Selection of soil treatments were informed by the fertilizer survey completed in Chapter 3. In selecting amendments, products verified to have a quantifiable gene content
across ARGs of interest. For the potting soil, a potting product found to contain either a low or unquantifiable gene content for the genes of interest was selected for inclusion in the present study as representative of a receiving soil source.

**Soil and Treatment Preparation**

Microcosms consisted of potting soil amended with 4 commercially available garden products verified to contain an ARG loading of the genes of interest. All treatments were prepared as directed by the manufacturer and incubated as a slurry at room temperature for 24 hours prior to day 0 to best homogenize the potting soil-treatment composite.

**Generating soil composites**

Four bags of potting soil were purchased and mixed by adding 1L of lightly packed soil from each bag into a 40L bucket have had been sterilized with ethanol just prior to soil addition. The composite was manually mixed for one minute prior to the addition of another 1L addition from each bag until a final volume of 12 liters was reached.

Treatments of manure, lawn amendment, food amendment and garden soil were prepared according to product guidelines, calculating for a soil depth of two inches and total volume of 2.5L (lightly packed). The manure, lawn amendment and garden soil products were added to the potting soil composite directly. Due to a low volume of treatment addition (according to product guidelines) the food amendment treatment was
ground in an ethanol sterilized ceramic pestle and mortar prior to addition to the potting soil to ensure a more even distribution of treatment.

**Microcosm assembly**

Initial microcosm assembly consisted of draining composite soils followed by loading soil-treatment composites into seed starter gardening wells. Seed starter wells were placed in garden trays, with separate garden trays used for each soil treatment.

Soil draining was achieved through a microcosm watering apparatus, which was assembled by suspending garden trays modified with 5/37’ drill holes above unmodified counterparts. Sterilized glass beakers were used to provide elevation between trays, allowing for the collection of runoff water.

*First flush*

Each treatment was drained by pouring the incubated slurry into the watering apparatus and manually mixing to ensure proper drainage. Drainage was defined as complete when at most one droplet of water would drain in a thirty second interval.

*Soil loading*

Sterilized glass beakers were used to lightly pack 40mLs of drained soil into seed-starter gardening wells, yielding a total of 48 wells per treatment (192 wells in total). Seed starter wells were placed in garden trays, with separate garden trays used for each soil treatment, yielding a total of 4 trays. Assembled garden trays were placed outside for a period of 24 days and sampled at three day intervals. Presented here are all even numbered time points.
**Microcosm Sampling**

Microcosm sampling consisted of watering soils followed by collection and processing of top soil (top 0.25 inches of soil) and bulk soil (depth of 1-1.5 inches below surface). (total n=432, n=192 for top soil and bulk soil each).

**Microcosm watering**

Each of the treatment trays were assembled into a watering apparatus (described above) and watered with deionized water. Watering volumes were dynamic and dependent on the number of wells at the time of watering. Volume of water used consisted of three times the soil volume in each tray, calculated as: 3*40mLs* n, where n is the number of wells loaded with a treated soil.

**Soil collection**

Upon watering, sampled were left to drain for approximately ten minutes, at which point destructive sampling was employed, with each treatment sampled in quadruplicate.

Wells were sampled at the surface to measure ARGs in soil most exposed to sunlight and potential solar degradation. Additional sampling occurred 1-1.5 inches into the well, a region still considered “surface soil” in the field of public health but not exposed to direct sunlight. Soil sampled 1-1.5in deep into the well is hereafter referred to as “bulk soil.”
Top soil at the surface of each well was sampled for ARG quantification by weighing 0.25 ± 0.01g of each well into individual 2mL screw cap tubes preloaded with 1 ± 0.05 g, 0.7mm garnet beads (Qiagen, Germantown, MD). After sampling completion, the first quarter inch of surface soil was removed and stored 4 °C for characterization. Following removal of the top quarter inch of soil, soil was scooped to remove the top 1-1.5 inches and sampled for ARG quantification as described above. Soil representing the “Bulk” region was stored at 4 °C for subsequent characterization.

**Sampling Conditions**

Sampling occurs between April and May of 2018 in Los Angeles, California. Rain was only encountered one day throughout the duration of the experiment. To avoid soil watering deviant from the designed experiment, samples were covered with a blue opaque tarp to shield from weather conditions. During this time, the samples were not exposed to direct sunlight.

**DNA Extraction**

All water and soil DNA extractions were completed using the DNeasy PowerSoil Kit (Qiagen, Germantown, MD) within three weeks of collection. Extractions proceeded per the manufacturer’s guidelines and an additional 2 mL screw cap tube preloaded with 1 ± 0.05 g of 0.7mm diameter garnet beads (Qiagen, Germantown, MD) and absent of sample (extraction blank) was extracted to identify contamination incurred during the extraction process should it occur. Resulting DNA was aliquoted and stored at -20 °C awaiting qPCR analysis. Total DNA concentration was determined by UV absorption via
a Nanodrop 2000C (Thermo Scientific, Waltham MA), as were 260/280 absorbance ratios.

**qPCR**

All samples were analyzed for ARG abundance of *sul1*, *ermF*, and *tetL* as well as *int1* and the 16S-rRNA gene, (a total bacteria surrogate measure) via qPCR. All Assays utilized PowerUp SYBR Green Master Mix and entailed a 25 µL reaction volume consisting of 12.5 µl of SYBR Green MasterMix (Life Technologies, Grand Island, NY), 1.25 µL of each primer, forward and reverse primer, and 2 µL of template DNA, with molecular grade water comprising any remaining reaction volume. Primers used were as developed and validated previously in literature.26–30 Primer sequences can be found Table 5-1, along with primer concentrations. Each assay run included a 7-point standard curve positive control, all applicable extraction blanks and a negative control of molecular grade water, with each sample plated in triplicate. All assays were performed in 96-well reaction plates using StepOne Plus (Applied Biosystems). Temperature cycles used can be found Table 5-1 and were as reported previously in literature.26–30
Table 5-1 | qPCR Primer Sequences Used

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer</th>
<th>Concentration</th>
<th>Sequence (5’-3’)</th>
<th>Annealing Conditions</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA</td>
<td>16S-rRNA-F</td>
<td>(100) nM</td>
<td>CCTACGGGAGGCAGCAG</td>
<td>60 °C/60 s</td>
<td>(27)</td>
</tr>
<tr>
<td></td>
<td>16S-rRNA-R</td>
<td>(100) nM</td>
<td>ATTACCGGCTGCTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sul1</td>
<td>sul1-F</td>
<td>(200) nM</td>
<td>CGCACCAGAACACATCGCAG</td>
<td>65 °C/60 s</td>
<td>(29)</td>
</tr>
<tr>
<td></td>
<td>sul1-R</td>
<td>(200) nM</td>
<td>TGAAGTCCCGCGCAAGCTCG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ermF</td>
<td>ermF-F</td>
<td>(500) nM</td>
<td>TCGTTTTACGCGTCACCTT</td>
<td>60 °C/30 s</td>
<td>(28)</td>
</tr>
<tr>
<td></td>
<td>ermF-R</td>
<td>(500) nM</td>
<td>CAACAAAGCCTGTCGTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tetL</td>
<td>tetL-F</td>
<td>(900) nM</td>
<td>GGTGGGAACGTCCTCATTACGT</td>
<td>60 °C/30 s</td>
<td>(31)</td>
</tr>
<tr>
<td></td>
<td>tetL-R</td>
<td>(900) nM</td>
<td>CCAATGGAAGGGCTACGCAAAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>intI1</td>
<td>intI1-F</td>
<td>(200) nM</td>
<td>GGTTCGTAGTGCCTGCTT</td>
<td>55 °C/30 s</td>
<td>(26)</td>
</tr>
<tr>
<td></td>
<td>intI1-R</td>
<td>(200) nM</td>
<td>CATCCCTGGCCGGTTTCT</td>
<td>72 °C/30 s</td>
<td></td>
</tr>
</tbody>
</table>

All samples were diluted to a concentration of 1 ng/µL prior to qPCR to offset inhibition effects. This chosen concentration was verified to effectively correct inhibition by well spike and serial dilution. For serial dilutions, extracted DNA was diluted to several concentrations to determine whether qPCR reaction inhibition occurrence. It was found that inhibition played a role in soil matrices and all samples were appropriately diluted. Efficacy of dilution to this concentration was confirmed by choosing at random and plating each sample into 6 qPCR wells. Three wells of each sample were spiked with known standard quantities. qPCR was performed with results confirming absence of inhibition effects with gene copy numbers consistently being equivalent to the sum of the spike quantity and unspiked sample. Dilution factors were back calculated during data analysis to obtain per gram quantification.
Target-containing DNA fragments to serve as positive controls were designed using sequences obtained from the NCBI database information and ordered through IDT Technologies. Known concentrations of the designed DNA fragment were run alongside environmental samples, yielding a seven-point standard curve and allowing for quantitation of gene copies. Standard curve efficiencies were 100 ± 6% across all qPCR assays and all $R^2$ values were at or above 0.99. Melt curves were used to further verify correct target gene amplification.

Results

Amendment selection

Garden amendments chosen for inclusion in this study were selected pursuant to the survey results in Chapter 3. One amendment was chosen from each major category reported in the survey: garden soil, food amendment, lawn amendment and manure, and one potting soil was selected as background soil. Brands from each category that reported consistent and quantifiable ARG copies were selected for inclusion in this study and are summarized in Table 5-2. The potting soil used was selected due to low ARG quantities, and initial ARG content was confirmed as described in Chapter 3.
Table 5-2 | Garden Products Selected for Microcosm Assembly

<table>
<thead>
<tr>
<th>Product Type</th>
<th>Product Name</th>
<th>Product brand</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potting Soil</td>
<td>Edna’s best potting soil</td>
<td>EB Stone</td>
</tr>
<tr>
<td>Garden Soil</td>
<td>Flower &amp; Vegetable Garden Soil</td>
<td>Sta Green</td>
</tr>
<tr>
<td>Food Amendment</td>
<td>Organic Plus: Tomato, Vegetable</td>
<td>Kellogg</td>
</tr>
<tr>
<td>Lawn Amendment</td>
<td>Turf Max</td>
<td>Green As It</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gets</td>
</tr>
<tr>
<td>Manure</td>
<td>Composted Chicken Manure</td>
<td>G&amp;B Organics</td>
</tr>
</tbody>
</table>

**ARG selection**

Genes selected for inclusion in this study have been suggested as indicators in assessing the antibiotic resistance status of the environment.\(^{34}\) These include the integron gene, \(int_1\), \(sul_1\) which confers resistance to sulfonamide, \(ermF\) which confers resistance to macrolides, and \(tetL\) which confers resistance to tetracyclines.

Mobile genetic elements including transposons,\(^{35}\) plasmids\(^{36}\) and integrons\(^{37,38}\) may play an important role in the dissemination and transfer of antibiotic resistance genes among and between organisms. In particular, the ability of integrons to capture and incorporate environmental ARGs into mobile cassettes via site-specific recombination makes integrons especially important in understanding horizontal gene transfer.\(^{39}\) The class 1 integron gene \(int_1\) has been proposed as a measure of anthropogenic pollution and additionally, has been frequently reported in the environment, making monitoring of the \(int_1\) gene of particular importance.\(^{40,41}\)
When applied as specified by the manufacturer, application of commercially sold manure proved to introduce the largest ARG input when compared to the other gardening treatments included in this study, resulting in an ermF loading of $5.37 \times 10^4$ copies per gram of soil (Figure 5-1). This is approximately one order of magnitude higher than the loading introduced by the selected garden soil and food amendment (average ermF loading $2.18 \times 10^3$ and $7.39 \times 10^3$ copies per gram of soil, respectively), and approximately four times the ermF load introduced by the lawn amendment product (average ermF loading of $1.26 \times 10^4$ gene copies per gram of soil).

**Figure 5-1 | ARG content in each soil-treatment composite at time zero.**
The ermF input resulting from garden soil application proved to exist below a detection limit set at 1 ermF copy per qPCR reaction. While soil treatment with the selected food amendment did introduce a quantifiable number of ermF gene copies, these copies underwent decay and were at levels below the detection limit prior to the completion of six days. Application of the included lawn amendment fell below the detection limit in the top soil but remained quantifiable in the bulk soil at the conclusion of 24 days. Manure treatment remained well over the detection limit for the duration of the microcosm experiment. (Figure 5-2)

While the garden soil and food amendment treatments underwent behavior demonstrative of decay, both manure and lawn amendment treatments exhibited a more complex temporal profile, altering between decay and proliferation. Lawn amendment bulk soil exhibited decay in the first 18 days of the experiment and proliferation between 18 and 24 days (an increase of approximately 5,000 copies per gram of soil). Overall, a 47% decrease in ermF loading was observed in bulk soil from day 0 to day 24. Manure treatment demonstrated a consistent increase in ermF loading for the first 12 days of the experiment (with a maximum average loading of $9.45 \times 10^4$ copies per gram of soil at twelve days, a 170% increase from the day 0), before decaying to 80% of the day 0 loading quantity in the bulk soil and to 65% in the top soil. (Figure 5-2)

Overall, application of garden products did not result in a quantifiable increase in ermF copy number in the top 0.25 inches of soil over a 24 time period, with the exception of manure application. Proliferation of the ermF gene load in bulk soil was confirmed to occur due to application of lawn amendment and, to a greater extent, application of the commercially available manure product.
**tetL**

Application of lawn amendment, food amendment and manure garden products introduced a quantifiable number of tetL while application of the garden soil product included in this study did not. Manure, food amendment and lawn amendment product inputs varied at the scale of 1-2 orders of magnitude, with input values measured at 2.6 x $10^5$, 1.8 x $10^3$, and 2.6 x $10^3$ gene copies, respectively. (Figure 5-3)

All products verified as a tetL input exhibited substantial decay with food and lawn amendment microcosms falling below the reliably quantifiable qPCR threshold by day 6. Manure amended microcosms sharply decayed by day 6, remaining quantifiable, but at 6% and 7% of the original input quantity in top and bulk soil, respectively. A brief period of proliferation yielded a maximum of 29% of the original input value in top soil after 12 days. Similarly, the bulk soil experienced a brief period of proliferation in tetL content, yielding a maximum of 28% of the original input value after 18 days. Proliferation was followed by decay and only 7% and 4% of the initial tetL input quantity remained at the conclusion of 24 days. (Figure 5-3)

Overall, only manure proved to be a tetL source over the 24 day experimental period. However, despite brief proliferation, manure inputs rapidly decayed, with a trend towards further decay should the 24 day timeline be extended.

**sul1**

Amendment application resulted in quantifiable copies of sul1 for each soil amendment included in this study. Gene loading quantities ranged from 2.7-5.4 x $10^4$ gene copies gram of soil following manure application, of 2.3 to 8.6 x $10^5$ gene copies
per gram of soil following garden soil application, 4.0 to 7.4 x 10^4 gene copies per gram following food amendment application and 1.4-2.8 x 10^4 copies per gram of soil following lawn amendment application. (Figure 5-1)

*sul1* gene copies were consistently quantifiable, at a detection limit above 1 gene copy per qPCR reaction across all time points. While *sul1* gene copies introduced via lawn amendment and garden soil amendment exhibited decay in *sul1* copies, both treatments continued to have quantifiable *sul1* quantities at the completion of 24 days. Top soil in garden soil microcosms had decayed by 63% from the initial at 24 days, with bulk soil having decayed by 58%. The lawn amendment microcosms exhibited a larger top oil and bulk soil disparity with top soil having only 14% of the initial *sul1* gene loading and bulk soil still containing 56% of the initial loading. (Figure 5-4)

Application of manure and food amendment resulted in more complex temporal profiles, with each treatment reporting both decay and proliferation of *sul1* quantities. Manure application resulted in ARG quantification as high as 1.8 x 10^6 gene copies per gram of bulk soil and as high as 1.5 x 10^6 gene copies per gram of top soil. These spikes were found to occur at 18 and 12 days, respectively. However, despite the sizeable spikes in gene content, topsoil and bulksoil displayed an overall decay by day 24, with topsoil going on to decay by 18% from initial loading and bulk soil decaying by 13%. Application of food amendment induced an ARG proliferation of up to 7.3 x 10^4 gene copies per gram in top soil while bulk soil was found to decay over the entire 24 day period. Overall, topsoil decayed to 35% of the initial *sul1* gene loading and bulk soil decay was quantified at 41% of the initial *sul1* gene loading at the completion of 24 days. (Figure 5-4)
Overall, manure application resulted in the largest quantifiable increase in sul1 across all time points, with all garden product application verifying an a quantifiable input of sul1 gene copies.

**int1**

Application of all treatments resulted in a quantifiable loading of the int1 gene, with manure application resulting in the highest int1 input (at an average $9.7 \times 10^4$ gene copies per gram of soil at day 0) (Figure 5-1). Lawn amendment application resulted in a loading of approximately $6.19 \times 10^4$ copies per gram of soil followed by food amendment application which resulted in $2.43 \times 10^4$ copies per gram of soil and finally garden soil application which resulted in a $2.15 \times 10^4$ copy per gram of soil gene loading. (Figure 5-1)

Garden soil, food amendment and lawn amendment treatments all underwent rapid decay that resulted in a drop to below the limit of detection by day 6 of the microcosm experiment. However, by day 12, all samples had begun to exhibit proliferation in both the top and bulk soil and were reliably quantifiable for the remainder of the experiment. Garden soil and food amendment treatments remained well below the initial int1 gene loading quantity with garden bulk soil exhibiting an overall 58% decrease in gene quantity between day 0 and day 24, and an overall 88% decrease in top soil. The food amendment treatment exhibited and overall 30% decrease in top soil and a 70% decrease in bulk soil. (Figure 5-5)

Lawn amendment application resulted in proliferation in the bulk soil well above the initial int1 loading (a 220% increase from day 0 to day 24), with the trend seen at day
suggesting that further proliferation may occur past the timeline of this experimental design. Top soil treated with lawn amendment remained above the limit of detection but displayed an overall decay of 56% from day 0 to day 24. Manure application resulted in rapid decay to below the detection limit at day 6, followed by rapid proliferation to 302% the initial $int_1$ loading in the top soil and 351% the initial gene quantity in bulk soil. Subsequent decay between day 12 and day 25 resulted in an overall 18% average decrease in $int_1$ gene quantity in top soil and an average 197% increase in gene quantity in bulk soil from day 0 to day 24. (Figure 5-5)

Overall, the $int_1$ gene was quantifiable in all soils following treatment application. Proliferation of the $int_1$ gene was confirmed to occur following use of lawn amendment. Proliferation was also confirmed following application of the manure product but was followed by rapid decay that resulted in a day 24 loading comparable to that of day 0. While garden soil and food amendment products showed trends suggesting subtle proliferation towards the end of the 24 day experiment, this increase in gene quantity remained substantially lower than the quantities measured at time 0 days.

$16s$-rRNA

When normalized by 16s-rRNA (a total eubacteria surrogate measure), patterns of proliferation and decay were found to persist, indicating that trends described cannot be attributed to fluxes in total bacteria. (Figure 5-6, Figure 5-7, Figure 5-8 and Figure 5-9)
**Overall observations**

ARGs do not necessarily follow a conventional decay pattern when appended to soil via commercially available gardening products. While application of garden soil and food amendment treatments were both confirmed as sources for all genes studied upon initial application (with the exception of tetL from garden soil), decay over 24 days prompted ermF and tetL to fall below the limit of detection set in this study. sul1 and int1 gene quantities resulting from garden soil and food amendment exhibited overall decay but remained reliably quantifiable upon experiment completion, with food amendment microcosms found to undergo both proliferation and decay in sul1 and int1 during the 24 day period.

Behavior proved to be more complex following manure and lawn amendment application. For all genes, manure microcosms verified fluctuations between decay and proliferation over the course of 24 days. Application of lawn amendment was particularly interesting with respect to changes in gene quantities, with ermF found to decay overall while int1 quantities increased substantially (by 220%) during the scope of this study. Application of commercially available manure proved to consistently provide the highest initial input of genes selected for this study. Additionally, with the exception of int1 in bulk soil, lawn amended microcosms, manure application consistently resulted in the highest quantity of gene introduction after 24 days, with final gene quantities ranging from 65% of the initial input to increases as high as 197%.

Nearly all microcosms confirmed a rapid decay in the first six days of the experiment. While behavior post-six days proved to be highly dynamic, results shown here suggest that soils undergo rapid changes in the first week following treatment.
application. However, despite this, monitoring for periods longer than an initial decay is necessary to observe potential rises in gene quantities over time. Of proliferation behavior reported here, proliferation can be seen to peak in the range of 12-18 days, with kinetics often switching to decay by the 24 day mark. This suggests that the first month of microcosm experiments are critical in understanding ARG kinetics following garden product application, with particular interest in the initial six days following application.

With the exception of food amendment application with respect to intI1, all microcosms hinted at a separation between bulk measurements and top soil. Top soil was found decay at a faster rate and generally reported values lower than those reported in bulk soil. These effects were most observed in the microcosms found to demonstrate quantifiable gene copy numbers for the duration of the 24 day experiment, indicating that these differences are most substantial when initial gene loadings are high. Further studies are needed to determine if differences between top soil and bulk soil may be due to solar degradation or shifts in the microbial community prompted by direct exposure to sunlight. What is suggested, however, is that if present, solar activation does not uniformly affect the topsoil region most relevant to public health.

While ermF quantities were consistently lower at 24 days than at day 0, intI1 quantities proved to be higher at the conclusion of this experiment for both manure and lawn amendment application, with lawn amendment suggesting even higher intI1 quantities beyond the time scope of this study. Additional studies are needed to determine whether an increase in intI1 is indicative of active horizontal gene transfer or conversely, if intI1 levels may indicate higher potential for and prompt an increase horizontal gene transfer, potentially leading to ARG proliferation beyond the initial 24 days.
Figure 5-2 | ermF copies per gram of garden product treated soil.
Figure 5-3 | tetL copies per gram of garden product treated soil.
Figure 5-4 | sul1 copies per gram of garden product treated soil.
Figure 5-5 | int1 copies per gram of garden product treated soil.
Figure 5-6 | ermF copies per 16s rRNA gene copies.
Figure 5-7 | tetL copies per 16s rRNA gene copies.
**Figure 5-8 | sul1 copies per 16s rRNA gene copies.**
Figure 5-9 | int1 copies per 16s rRNA gene copies.
References

(1) Organization, W. H. Global action plan on antimicrobial resistance. 2015.


(8) Chen, C.; Li, J.; Chen, P.; Ding, R.; Zhang, P.; Li, X. Occurrence of antibiotics and


(16) Heuer, H.; Solehadi, Q.; Zimmerling, U.; Kleineidam, K.; Schloter, M.; Müller, T.; Focks,


(22) Burch, T. R.; Sadowsky, M. J.; Lapara, T. M. Fate of Antibiotic Resistance Genes and Class 1 Integrons in Soil Microcosms Following the Application of Treated Residual Municipal Wastewater Solids Fate of Antibiotic Resistance Genes and Class 1 Integrons in Soil Microcosms Following Current affiliati. **2014**.

(23) Wang, M.; Liu, P.; Xiong, W.; Zhou, Q.; Wangxiao, J.; Zeng, Z.; Sun, Y. Fate of potential


(39) Alekshun, M. N.; Levy, S. B. Molecular Mechanisms of Antibacterial Multidrug


Chapter 6: Summary and Conclusions

The work presented here seeks to understand the quantity and dynamics of ARGs in places and environmental compartments that share an intimate connection with the human populations—the parks we play in, the air we breathe, the tap water we drink and the soil that we use to plant food.

These studies confirm that ARG levels are disparate, ubiquitous and cannot be directly attributed to either urbanization or agriculture, rather, there are variables much more complex that dictate ARG persistence, proliferation and decay.

In chapter two, it was concluded that unique ARG profiles will exist in the drinking water, soil and air of any given location. Additionally, within a region the size of California, cities can and will exhibit unique relationships to ARGs.

Chapter three verified the role of confined animal feeding operations as sources of airborne antibiotic resistance, indicating a mechanism for ARG dissemination over which residents have no control. Additionally, third party organic verification proved to be an inadequate predictor for mitigating ARG content.

Chapter four and five verify commercially available products as sources of antibiotic resistance genes, highlight a lack of transparency regarding manure source and treatment prior to distribution, and demonstrate that dynamic changes in ARGs introduced through gardening products occur over time frames as short as one month.