November 2017

The Ecology of Antibiotic Resistance: Sources and Persistence of Vancomycin-Resistant Enterococci and Antibiotic Resistant Genes in Aquatic Environments

Suzanne M. Young
University of South Florida, suzanneyoung@mail.usf.edu

Follow this and additional works at: http://scholarcommons.usf.edu/etd

Part of the Aquaculture and Fisheries Commons, Environmental Sciences Commons, and the Microbiology Commons

Scholar Commons Citation

This Dissertation is brought to you for free and open access by the Graduate School at Scholar Commons. It has been accepted for inclusion in Graduate Theses and Dissertations by an authorized administrator of Scholar Commons. For more information, please contact scholarcommons@usf.edu.
The Ecology of Antibiotic Resistance:
Sources and Persistence of Vancomycin-Resistant Enterococci and Antibiotic Resistance Genes
in Aquatic Environments

by

Suzanne M. Young

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Integrative Biology
College of Arts and Sciences
University of South Florida

Co-Major Professor: Valerie (Jody) Harwood, Ph.D.
Co-Major Professor: Jason Rohr, Ph.D.
James Riordan, Ph.D.
Kathleen Scott, Ph.D.

Date of Approval:
October 23, 2017

Keywords: Antimicrobial resistance, water quality, public health, microbial ecology, sewage contamination, mesocosms

Copyright © Suzanne M. Young 2017
DEDICATION

To my family, whose lived examples of dedication, perseverance, pursuit of knowledge, love for the planet and love for humanity have guided me to be who I am and accomplish what I have. To Dad, charismatic, kind, pragmatic, thoughtful, loving, driven, proud of me every day I can remember. To Mom, forever forgiving, stoically brave, relentless, unflinchingly moral, generous, unstoppable. To Dr. Nancy Bishop, Dr. Ronald Bishop, Arthur Young and Luciana Young, always unwavering in support and love, instilling in me the importance of education, service and community. And especially to Nana, only a few credits away from her doctorate at age 90 after raising 3 kids and earning credits at multiple universities, always a doctor to me. To Erica, Will, Everett and Emmy, because being Aunt Suz is my favorite thing to be. To my chosen family in Tampa, New York, California, Scotland, Kenya, and beyond, for keeping me grounded and sane. Rahul told me not to take it personally and to never give up. Fernanda inspired me to persevere. Taegan showed me work-life balance. Megan showed me the other side. Marcel gave me perspective. To Jennifer’s laughter, Emma’s honesty, Jessica’s poetry. To Dr. Eva Chase, whose spirit and speed continue to inspire me. To women in science, those who speak out and those who don’t, those who fight against racism, sexism and the perpetuation of stereotypes, those more courageous than me and those with more to lose. To my union, Graduate Assistants United, and to workers everywhere. Solidarity.
ACKNOWLEDGEMENTS

The completion of this degree would not be possible without the support of my academic advisers, Dr. Valerie (Jody) Harwood and Dr. Jason Rohr and members of my committee, Dr. KT Scott and Dr. James Riordan. The EPA STAR Graduate Fellowship provided funding for my research for two years. Former and current members of the Harwood and Rohr Labs, especially Dr. Bina Nayak and Dr. Pauline Wanjugi, provided major support. Lab assistance from Scott Bessler, Jenna Hindsley, Aldo Lobos, James Conrad and Jacob Senkbeil was essential and appreciated. Dr. Guido Werner generously provided the bacterial strains used in Chapter 4. Dr. Lisa Durso advised on antibiotic resistance gene testing in poultry litter microcosms.
TABLE OF CONTENTS

LIST OF TABLES .................................................................................................................................................. iii

LIST OF FIGURES .................................................................................................................................................. iv

ABSTRACT .............................................................................................................................................................. v

CHAPTER 1: BACKGROUND AND REVIEW OF ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT ................................................................................................................................. 1
  INTRODUCTION .................................................................................................................................................. 1
  EVOLUTION AND MOLECULAR FOUNDATIONS ................................................................................................. 4
  ANTIBIOTIC USE IN ANIMAL AND HUMAN HEALTH ...................................................................................... 7
  MONITORING ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT: METHODS OF DETECTION AND EXPLORATORY STRATEGIES .................................................................................. 9
  PREVALENCE OF ANTIBIOTIC-RESISTANT BACTERIA AND ANTIBIOTIC RESISTANCE GENES IN THE ENVIRONMENT ............................................................................................................. 11
    Urban stormwater and sewage contamination ................................................................................................ 12
    Animal agriculture ........................................................................................................................................... 13
  VANCOMYCIN-RESISTANT ENTEROCOCCI ....................................................................................................... 14
  RESEARCH CHAPTERS: GOALS OF THE DISSERTATION ................................................................................. 17
  REFERENCES ....................................................................................................................................................... 18

CHAPTER 2: VANCOMYCIN-RESISTANT ENTEROCOCCI AND BACTERIAL COMMUNITY STRUCTURE FOLLOWING A SEWAGE SPILL INTO AN AQUATIC ENVIRONMENT (SEE APPENDIX B) .................................................................................................................. 32

CHAPTER 3: PERSISTENCE OF ANTIBIOTIC RESISTANCE GENES FROM POULTRY LITTER IN AQUATIC ENVIRONMENTS ........................................................................................................... 33
  ABSTRACT ......................................................................................................................................................... 33
  BACKGROUND .................................................................................................................................................. 34
  MATERIALS AND METHODS ............................................................................................................................. 39
    Microcosm Set-up .......................................................................................................................................... 39
    Sampling Procedures .................................................................................................................................... 39
    Detection of Antibiotic Resistance Genes by PCR and qPCR .................................................................... 40
    Analysis ....................................................................................................................................................... 40
  RESULTS ............................................................................................................................................................ 41
    Detection by PCR ......................................................................................................................................... 41
    Measurement by qPCR .............................................................................................................................. 41
  DISCUSSION ...................................................................................................................................................... 46
  REFERENCES ...................................................................................................................................................... 50
CHAPTER 4: NUTRIENT-DEPENDENT EFFECT OF PLASMIDS CONFERRING HIGH-LEVEL VANCOMYCIN RESISTANCE ON PERSISTENCE OF ENTEROCOCCUS FAECIUM IN AMBIENT WATERS ........................................ 58

ABSTRACT .................................................................................................................. 58

BACKGROUND .......................................................................................................... 59

MATERIALS AND METHODS .................................................................................... 61

Enterococcus faecium strains .................................................................................... 61
Microcosm Set-up .................................................................................................. 62
Sampling Collection and Processing .................................................................... 63
Data Analysis .......................................................................................................... 64

RESULTS .................................................................................................................. 64

DISCUSSION ............................................................................................................ 69

REFERENCES .......................................................................................................... 73

AFTERWORD ............................................................................................................. 79

APPENDICES ............................................................................................................ 82

APPENDIX A. TABLE 1. ANTIBIOTICS RELEVANT TO HUMAN AND ANIMAL HEALTH ...... 82

APPENDIX B. CHAPTER 2 (PUBLICATION) ................................................................. 84
LIST OF TABLES

CHAPTER 3: PERSISTENCE OF ANTIBIOTIC RESISTANCE GENES FROM POULTRY LITTER IN AQUATIC ENVIRONMENTS

Table 1. PCR Conditions for PCR and qPCR Assays ........................................... 45
Table 2. Persistence of antibiotic resistance genes by PCR .............................. 45

CHAPTER 4: NUTRIENT-DEPENDENT EFFECT OF PLASMIDS CONFERING HIGH-LEVEL VANCOMYCIN RESISTANCE ON PERSISTENCE OF ENTEROCOCCUS FAECIUM IN AMBIENT WATERS

Table 1. E. faecium strain origin and plasmid characteristics .......................... 66

APPENDIX A

Table 1. Antibiotics relevant to human and animal health .............................. 82

APPENDIX B

Table 1. Detection of vanA in water and sediment ........................................... 88
LIST OF FIGURES

CHAPTER 3: PERSISTENCE OF ANTIBIOTIC RESISTANCE GENES FROM POULTRY LITTER IN AQUATIC ENVIRONMENTS

FIGURE 1. CONCENTRATIONS IN MICRO COSMS OVER TIME ............................................. 43
FIGURE 2. DECAY OF ANTIBIOTIC RESISTANCE GENES ................................................ 44

CHAPTER 4: NUTRIENT-DEPENDENT EFFECT OF PLASMIDS CONFERRING HIGH-LEVEL VANCOMYCIN RESISTANCE ON PERSISTENCE OF ENTEROCOCCUS FAECIUM IN AMBIENT WATERS

FIGURE 1A. MEAN CONCENTRATIONS OF E. FAECIUM WITHOUT ADDED NUTRIENTS ...... 67
FIGURE 1B. MEAN CONCENTRATIONS OF E. FAECIUM WITH ADDED NUTRIENTS .......... 67
FIGURE 2. COMPARISON OF REDUCTION FOR EACH E. FAECIUM STRAIN ...................... 68
FIGURE 3. CORRELATIONS OF PLASMID SIZE AND REDUCTION OF E. FAECIUM .......... 68

APPENDIX B

FIGURE 1. CULTURABLE ENTEROCOCCI IN WATER AND SEDIMENT .................................. 87
FIGURE 2. ANALYSIS OF DNA SEQUENCING ................................................................... 88
FIGURE 3. RELATIVE ABUNDANCE OF SEWAGE ASSOCIATED OTU’S ............................. 88
FIGURE 4. DOMINANT FAMILIES IN SEDIMENT AND WATER ........................................ 89
FIGURE S1. MAP OF STUDY AREA .................................................................................... 93
FIGURE S2A. MAP OF STUDY AREA: ADDITIONAL SAMPLING SITES ......................... 94
FIGURE S2B. MAP OF STUDY AREA: ADDITIONAL SAMPLING SITES ......................... 95
FIGURE S3A. PHOTO LOOKING EAST ................................................................................ 96
FIGURE S3B. PHOTO LOOKING WEST ............................................................................... 96
FIGURE S4A. TAXONOMIC DIVERSITY DATA IN WATER ................................................ 97
FIGURE S4B. TAXONOMIC DIVERSITY DATA IN SEDIMENT ........................................ 97
FIGURE S5A. RELATIVE ABUNDANCE OF FAMILIES ASSOCIATED WITH ENTERIC PATHOGENS IN WATER .................................................................................. 98
FIGURE S5B. RELATIVE ABUNDANCE OF FAMILIES ASSOCIATED WITH ENTERIC PATHOGENS IN SEDIMENT .............................................................. 98
ABSTRACT

The growing crisis of antibiotic resistance is a major threat to ecosystems and human health. Infections caused by known and emerging antibiotic resistant pathogens are on the rise globally, with approximately 700,000 deaths per year caused by antibiotic resistant bacteria (1). In the United States, infections from antibiotic resistant bacteria cause more than 2 million illnesses and 23,000 deaths (2). Antibiotic resistant bacteria and antibiotic resistance genes are released into aquatic ecosystems through hospital waste, residential sewer lines and animal agricultural waste streams. Animal agriculture accounts for approximately 70% of antibiotic use in the United States (3). In agricultural ecosystems, runoff, land-applied fertilizer and waste lagoons can all contribute to the spread of antibiotic resistance. In urban ecosystems, sewage spills and other wastewater inputs contribute to the spread of antibiotic resistance. Environmental matrices, such as soil and water, can provide habitat, serving as reservoirs to potentially promote the spread of resistance. Research addressing antibiotic resistance primarily focuses on monitoring clinical occurrence and nosocomial infections (acquired in hospitals), but the natural environment also plays a role in the spread of antibiotic resistance. The consequences to aquatic ecosystems are not often studied and not well understood. Antibiotic resistance genes can transfer between bacteria through transduction, transformation and conjugation, potentially persisting in non-pathogenic environmental bacteria. Environmental reservoirs of antibiotics, antibiotic resistant bacteria and antibiotic resistance genes should be considered and integrated
into frameworks to improve monitoring, regulation and management of urban and rural watersheds.

The research presented in this doctoral dissertation includes field and laboratory studies designed to assess the prevalence and persistence of antibiotic resistant bacteria and antibiotic resistance genes in aquatic environments, with a focus on vancomycin-resistant enterococci, which are considered a major threat in the United States and top priority pathogens according to the Centers for Disease Control (2). The *vanA* gene associated with high-level resistance is located on mobile plasmids and associated with clinical infections, predominantly in the species *Enterococcus faecium*. *E. faecium* can cause bacteremia, endocarditis, pelvic infections and more (4). When vancomycin, often the last line of treatment for these infections, is no longer effective, the health burdens increase both financially and physically and infections can be fatal.

Chapter 1 summarizes background and review of antibiotic resistance in the environment, including a co-authored review of culture-based methods to detect antibiotic resistant bacteria and antibiotic resistance genes in in the environment (previously published in the Journal of Environmental Quality (5)). In Chapter 2, a field study was performed to investigate the occurrence and persistence of vancomycin-resistant enterococci and *vanA* in a sewage spill in Pinellas County, Florida, previously published in the journal *Applied and Environmental Microbiology* (6). In Chapter 3, antibiotic resistance genes were quantified to study their persistence in poultry litter microcosms (manuscript in prep). In Chapter 4, microcosms were used to assess how nutrients and plasmid-associated vancomycin resistance affect survival among *E. faecium* strains (in process of submitting for publication at *Applied and Environmental Microbiology*).
Antibiotic resistance is a public health crisis and the results of the studies presented here contribute data towards a better understanding of environmental reservoirs of antibiotic resistant bacteria and antibiotic resistance genes. The research has broad implications for public health, environmental policy and ecosystem management.
CHAPTER 1: BACKGROUND AND REVIEW OF ANTIBIOTIC RESISTANCE IN THE ENVIRONMENT

Introduction

The quest to discover and develop compounds to treat human disease is as ancient as the history of medicine, but developments resulting from scientific research propelled major medical advancements in the early 20th century. Some of the earliest efforts in drug discovery were led by Paul Erlich beginning in 1904, in the search for treatment of syphilis (7). One of Erlich and his collaborators’ first successes was the development and distribution of Salvarsan, an organic arsenical, which has since been used in cancer treatment (8), followed by discovery of sulfa drugs, more commonly known as sulfonamides (9). Another scientist, Selman Waksman, discovered more than 20 antibiotic compounds beginning in 1910, including streptomycin and neomycin, by studying Actinomyces spp. and the compounds these soil bacteria produced (10). Perhaps the most famous milestone in the history of antibiotics occurred in 1929, when Alexander Fleming observed mold contamination produce a compound that prevented the growth of bacterial cultures on agar plates (11), a compound that was eventually characterized as penicillin.

Since the early 20th century, overuse of antibiotics in both human and animal health combined with the slowed pace in novel drug development has led to the spread of antibiotic resistance in pathogenic bacteria. Penicillin was introduced to the American market in the 1940’s, but resistance to the drug was reported almost immediately after its implementation in
healthcare settings (7). This pattern of rapid development of antibiotic resistance in bacteria has been observed with most new antibiotics in the 20th century, contributing to the current global public health crisis (12). Each year, millions of illnesses and thousands of deaths are caused by bacterial infections that are not responsive to traditional antibiotic therapy (2). The evolutionary mechanisms that contribute to antibiotic resistance on a cellular level include horizontal gene transfer and selective pressure resulting in natural selection of resistant strains.

Technological and scientific developments have increased our understanding of antibiotic resistance, but antibiotic resistance is an ancient and natural phenomenon, as evidenced by more recent discoveries of antibiotic resistant bacteria and antibiotic resistance genes in ice cores, caves and glaciers (13-15), tetracycline in early human civilization (16), and antibiotics derived from environmental sources including soil bacteria and marine life (17, 18). However, antibiotic resistance is an increasingly urgent problem, in that the engineering of new drugs has not kept pace with resistance to old ones (19). Growing difficulties in novel drug discovery combined with the lack of financial incentive for the pharmaceutical industry has contributed to the antibiotic resistance problem, and innovative approaches using more advanced technology are now being explored to determine new modes of disrupting infections caused by antibiotic resistant bacteria (20, 21).

Many public health and scientific research agencies have called for action to prevent the spread of resistance and to study the crisis as it unfolds, including the Centers for Disease Control (CDC), National Institute of Health (NIH) and Environmental Protection Agency (EPA). In 2016, the United Nations held its first special session on a health-related issue since the AIDS crisis in the 1990’s, describing antibiotic resistance as the most urgent public health crisis the world faces (22). The prospective future of a world without antibiotics, or a “post-antibiotic
era” (23-25) has energized political and scientific investments to address the antibiotic resistance problem. In the developed world, most transmission of infections caused by antibiotic resistant bacteria occurs in hospitals (nosocomial), while areas with poor sanitation infrastructure also have to combat community-level transmission.

Animal and human waste are the dominant sources of antibiotics, antibiotic resistant bacteria and antibiotic resistance in the environment, released through runoff and sewage discharges (26, 27). Most antibiotic compounds are not fully processed or broken down by animal metabolism after consumption or administration and are excreted as waste (28). The detection of antibiotic resistance determinants outside of hospitals has been reported in wildlife feces (29-31), groundwater (32-34), freshwater and marine environments (34-40), drinking water (41), and wastewater treatment plants (42-46). Challenges remain in studying how antibiotic resistance spreads in the extraintestinal environment, especially with how to effectively choose which genes, bacteria or antibiotics to target given the natural history of antibiotics.

The public health risks of antibiotic resistance in the environment remain unclear, with gaps in knowledge and challenges in communicating potential risk to the public. Major research needs include longitudinal surveillance in environmental reservoirs, empirical studies of antibiotic resistant bacteria and antibiotic resistance genes outside of clinical settings and epidemiological studies to quantify human health risks. Laboratory studies are necessary to understand mechanisms of resistance, survival of antibiotic resistant bacteria in extraintestinal environments and rates of horizontal gene transfer. Empirical studies and modeling approaches will contribute to the foundational understanding of antibiotic resistance in the environment and can inform management and regulatory policy, protecting ecosystems and public health.
Evolution and Molecular Foundations of Antibiotic Resistance

Natural selection is a driving force behind the evolution of antibiotic resistance, including the spread of resistance in the environment (47). Random mutations can contribute to the spread of resistance, where selective pressure can increase the survival of organisms harboring antibiotic resistance genes (48). Selective pressure allows antibiotic resistant bacteria to survive and outcompete susceptible organisms and genes that confer antibiotic resistance to proliferate (49, 50). These genes can spread throughout populations and communities by horizontal gene transfer, a major mechanism of evolution in prokaryotic adaptation and diversification (51-55). Such adaptation can occur in direct response to antibiotic exposure or indirectly, as mutations in certain housekeeping genes render some antibiotics ineffective (56). Resistance can also come with a cost, and studies have shown plasmids or other resistance mechanisms can be detrimental to bacterial fitness (57, 58). The evolution of antibiotic resistance has been studied extensively, but research continues to develop regarding evolutionary mechanisms of resistance (59, 60).

Many antibiotic resistance genes are located on plasmids, or associated with transposons, integrons and gene cassettes, mobile genetic elements that are necessary for horizontal gene transfer (61). Gene acquisition through horizontal gene transfer diversifies bacterial populations through structural genomic changes that occur at individual levels. Horizontal gene transfer occurs laterally, can be interspecific and accounts for a majority of the plasticity in evolution of bacterial genomes (62, 63). Horizontal gene transfer has been widely described and scientifically supported as a key mechanism of prokaryotic and eukaryotic evolution (52, 53, 64-66). For example, genomic sequencing analyses of *Saccharomyces cerevisiae* revealed that various genes, including one for the enzyme dihydroorotate dehydrogenase, were derived from bacterial genomes and acquired through horizontal gene transfer (67). Horizontal gene transfer has
contributed to the spread of antibiotic resistance in human pathogens, through the transfer of
virulence genes and antibiotic resistance genes (63, 68-70). The study of *Staphylococcus aureus*
and the mobile cassette chromosome *mec* (*SCCmec*), responsible for methicillin resistance,
demonstrate the ways in which horizontal gene transfer affects human health by spreading
antibiotic resistance among pathogens (71-73).

Plasmids are extrachromosomal genetic structures within bacteria, characterized by their
size, copy number, and the genes they carry for replication and transfer, among other functions
(74). Plasmids can also contain integrons, mobile genetic elements that carry antibiotic
resistance genes and insertion sequences for site-specific integration (75-77). Plasmid-borne
antibiotic resistance genes can be transferred between bacteria in environmental reservoirs
through conjugation, including transfer to non-pathogenic endogenous bacteria, potentially
establishing environmental reservoirs of transferable antibiotic resistance genes (78).

Conjugative antibiotic resistance plasmids are rarely assessed in realistic, dynamic ecosystems
because quantifying rates of plasmid transfer outside of nutrient-rich media is challenging, with
transfer rates reported from $10^{-5}$ to $10^{-14}$ transfers per cell (79). However, because plasmids
represent a major vehicle for gene transfer in aquatic ecosystems, these research challenges need
to be pursued.

Plasmid maintenance is regulated by a variety of genetic factors that can also influence
general microbial fitness, including post-segregational killing mechanisms (74). Post-
segregational killing mechanisms prevent survival of daughter cells that do not contain the parent
plasmids, enabling plasmid-bearing strains to persist and succeed in diverse environments (80).
When post-segregational killing systems are absent, and in the absence of selective pressure by
antibiotics, plasmid maintenance is reduced (81, 82).
Antibiotic resistance plasmids have not been studied extensively in terms of nutrient-dependent effects on survival or replication, but research has shown that plasmids can impact survival, and that different plasmid types, systems and bacterial hosts can have varying effects (57, 83). The metabolic expense of maintaining plasmids, especially large plasmids, has been associated with a cost to fitness in various classes of plasmids and bacteria (84, 85). Nutrient-dependent costs have been observed with virulence plasmids in *Escherichia coli* (86) and *Agrobacterium tumefaciens* (87), where nutrient abundance was generally associated with a reduced plasmid burden (i.e., higher nutrient levels were beneficial to plasmid-bearing strains). Plasmids of *Haemophilus influenza* were shown to adapt to antibiotic addition treatments in culture, where plasmid copy number and fitness contributed to enhanced antibiotic resistance, and with results also suggesting that only newly acquired antibiotic resistance plasmids presented a cost to fitness (88). In a separate study, large plasmids were shown to have greater cost to fitness than smaller plasmids in vancomycin-resistant *Enterococcus* spp. (85). Few studies have assessed fitness in an ecological context, outside of nutrient-rich environments and in terms of reservoirs of antibiotic resistance in aquatic ecosystems.

Transduction and transformation are also modes of horizontal gene transfer by which antibiotic resistance genes can spread (89). Transduction is the transfer of genes through viral vectors and can contribute to the spread of antibiotic resistance, primarily through bacteriophage (90-92). Transfer of antibiotic resistance genes through phage has been reported in pathogens relevant to environment and human health, including *Enterococcus* spp. and *Staphylococcus aureus* (93, 94), and phages carrying antibiotic resistance genes have been detected in wastewater (95). Recent bioinformatics analysis has shown that phages are far less likely to

---

1 Plasmids of vancomycin-resistant enterococci are discussed further in Chapter 4 of this dissertation.
carry resistance genes than previously thought (96), but studies have shown that phages do have the ability to carry and transfer antibiotic resistance genes (90, 97). DNA transformation has also been demonstrated as a mode of antibiotic resistance gene transfer (61, 98) but is much less studied in ecological contexts and requires further empirical research in the context of the spread of antibiotic resistance in the environment. Most research regarding uptake of free DNA is related to the use of antibiotic resistance genes in transgenic plants and the potential for transformation by pathogens (99).

**Antibiotic Use in Animal and Human Health**

Antibiotic compounds target specific biochemical or physiological processes of cell reproduction and metabolism (100, 101), such as inhibiting cell wall synthesis in many Gram-positive bacteria in the case of vancomycin (102, 103). Antibiotic compounds are effective because they target structures absent from or fundamentally different in hosts vs. pathogens. Antibiotic resistance genes encode capabilities to counteract the effects of antibiotics, and are often used as target determinants of antibiotic resistance in environmental reservoirs. Table 1 includes a summary of common antibiotics, modes of actions and resistance genes that are relevant in the research covered in this dissertation (see Appendix A).

Industrialization of animal husbandry has led to increased use of therapeutic drugs as prophylactics, for growth promotion and infection prevention (104, 105). It is estimated that 70% of antibiotics bought in the United States are used in animal agricultural systems, including use as prophylactics or for growth promotion (3). Animal-based food production in developed countries has shifted largely to concentrated animal farming operations where animals are often kept in unsanitary conditions, requiring increases in antibiotic applications to prevent the spread of disease (106). This is the case for bovine, swine, poultry and aquaculture, and waste animal
farming contributes to the spread of antibiotics and antibiotic resistant bacteria in the environment (100, 107-110). Many farming operations utilize waste lagoons to partially treat or settle waste before discharging into nearby surface waters, which can also reach sediments, soil and groundwater (37, 111). In addition, the use of land-applied manure can increase the spread of antibiotic resistant bacteria and antibiotic resistance genes from animal agriculture to the wider environment and potentially crops for human consumption (112-114)\(^2\). Federal legislation to restrict antibiotic use in animals has also been introduced, but the U.S. government has been slow to regulate or restrict antibiotic use in animal agriculture (115).

Many medical organizations and scientific societies encourage governmental action to reduce antibiotic prescription rates in human health applications (116), particularly in the United States with approximately 250 million prescriptions per year (117). The prudent use and prescription of antibiotics is key to preventing the further spread of resistance; one study from 2009 showed that at least 30% of prescriptions were unnecessary (118), based on prescribing and institutional guidelines (119). Stewardship initiatives have been pursued and implemented in recent years, but experts say that resources to support these initiatives are still lacking, citing the need for international staffing standards and sustainable funding (120). A group of federal agencies (Centers for Disease Control and Prevention, Federal Drug Administration, United States Department of Agriculture) formed the National Antimicrobial Resistance Monitoring System for Enteric Bacteria (NARMS) in 1996 (121), designed to improve surveillance and reporting mechanisms. While monitoring and surveillance in hospitals have become standard

\(^2\) Links between the animal agricultural applications of antibiotics and human health impacts have been demonstrated with the use of glycopeptide antibiotics in 1990’s Europe leading to an increase in human infections from vancomycin-resistant enterococci (VRE); for more information on VRE and avoparcin, see section on VRE in this chapter.
practice, the problem persists. In 2010, average prescription rates in the United states were as high as 1,239 per 1,000 patients, based on analysis of state-specific data (12).

**Monitoring Antibiotic Resistance in the Environment: Methods of Detection and Exploratory Strategies**

Until very recently, the study of antibiotic resistance in environmental reservoirs has been carried out primarily by culturing pathogens and testing for susceptibility to various antibiotic compounds relevant to human or animal health. These culture-based antimicrobial susceptibility testing strategies include disk diffusion, broth dilution, agar plate dilution and automated methods including VITEK and Sensititre systems that use fluorometry for clinical diagnosis (5). Cultivating bacteria is an effective method in assessing whether bacteria are viable and to test the limits of resistance to different drugs, but can be time consuming and limited based on recovery of only culturable microbes.

Molecular methods that identify and target specific antibiotic resistance genes are now common in environmental settings, using PCR and qPCR as well as metagenomic analyses (122). Recent technological advancements in molecular methods allow for the targeting of resistance genes in environmental samples and resistome assessments based on Illumina or next generation sequencing (5, 123-126). These advances have also led to the discovery and targeting of many mobile resistance elements, including transposons and integrons, which carry resistance genes and are readily transferred among bacterial species.

---

Currently, there are no established methods or regulatory guidelines for monitoring antibiotic resistance in the environment outside of hospital settings. Many bacteria are intrinsically resistant to some antibiotics (127) and in an environmental context, antibiotic resistance determinants do not always translate to public health risks. Therefore, it is ineffective to indiscriminately monitor antibiotic resistant bacteria or antibiotic resistance genes as direct proxies for pollution or human health risks. Antibiotic resistance genes are abundant and diverse, found in almost any soil sample because soil microbes naturally produce antibiotic compounds (128). Fecal indicator bacteria, such as *E. coli* and enterococci, have been established as reliable indicators of human health risk in recreational waters based on their association with gastrointestinal illness (129-131). These bacteria in conjunction with antibiotic resistance genes may also serve as useful proxies for assessing the spread of antibiotic resistance in aquatic environments, especially related to sewage contamination.

Monitoring background levels of antibiotic resistance targets in both pristine and polluted areas using spatial and temporal sampling is also crucial in studying antibiotic resistance in the environment (132, 133). One study in German and Australian surface waters showed frequent detection of sulfonamide resistance genes in their assessment of 24 antibiotic resistance genes, emphasizing the role of aquatic ecosystems as reservoirs (134). Another broad survey in China found over 200 antibiotic resistance genes in 18 estuaries, with a strong connection to human development (135).

Most studies determine genetic or bacterial targets for measuring antibiotic resistance based on sample site and probable sources of contamination. For example, in tracking antibiotic resistance genes in and around wastewater treatment plants, specific genes are targeted that are associated with resistance to pharmaceuticals used in human health. Common targets in recent
studies assessing human impacts in environmental matrices include mobile genetic elements such as Class 1 integrons (136-138) and genes conferring resistance to various classes of antibiotics commonly used in hospitals including sulfonamides, tetracyclines and β-lactams (139-143). In animal agricultural ecosystems, researchers target genes associated with antibiotics used in animal farming as well as common pathogens frequently found in animal waste. When investigating sites in proximity to poultry farming operations, common methods include isolating pathogens associated with that industry, such as *Salmonella* spp. and *Campylobacter* spp., and testing isolates for susceptibility to relevant classes of antibiotics (144-148).

The complex nature of natural ecosystems contributes to the difficulty in monitoring antibiotic resistance in the environment, including the variability in environmental matrices and the role of environmental factors in gene expression and modulation (149). Bacteria can have different rates of decay in different types of substrates including sediment, water, vegetation and attachment to other particles, microbes, copepods and organic material (28, 44, 150-155). Fecal indicator bacteria (enterococci) have been shown to attach to particles variably based on microenvironment and level of sewage contamination (156). Selective pressure in aquatic ecosystems from chemicals such as antibiotics and metals can also contribute to the proliferation of resistance traits (157-159).

**Prevalence of Antibiotic-Resistant Bacteria and Antibiotic Resistance Genes in the Environment**

The release of antibiotic resistant bacteria and antibiotic resistance genes into the environment from anthropogenic sources can contribute to environmental reservoirs. The
prevalence of antibiotic resistant bacteria and their genes in predominant sources (animal and human waste, stormwater, sewage) are reviewed and summarized below.

*Urban stormwater and sewage contamination*

Stormwater is the mix of rainwater and sewage that can release sewage and its associated microbes into waterways in communities with both combined and separated sanitary waste systems. Infrastructure capabilities, storage demands during heavy rainfall and poorly maintained sewer systems contribute to the problem of surface water quality. Excess volumes of rainfall add to sewage already flowing to wastewater treatment plants, sometimes leading to discharge or overflow events and infiltration through leaky sewer pipes. Water quality guidelines for recreational water use do not include antibiotic resistance determinants, but many studies have examined how sewage, stormwater and wastewater treatment plants can contribute to the spread of antibiotic resistance in the environment, through detection and quantification of antibiotic resistant bacteria and antibiotic resistance genes.

Studies worldwide have consistently identified sewage and stormwater as sources of antibiotic resistant bacteria and resistance genes in waterways, including detection in lakes in China (160) and seawater in Greece (161). Antibiotic resistant bacteria and antibiotic resistance genes have been detected in surface water impacted by stormwater and sewage using culture-based and molecular methods (162-164). Research using experimental wetlands has also confirmed stormwater as a significant source of antibiotic resistance genes in aquatic environments (165).

Clinically relevant antibiotic resistant bacteria and antibiotic resistance genes are frequently detected in association with urban development and wastewater treatment effluent (166-172). Treatment processes and the level of disinfection can impact survival of antibiotic
resistant bacteria and proliferation of antibiotic resistance genes (173). In municipal wastewater treatment, studies have shown that selection for antibiotic resistant *Acinetobacter* spp. (174) and increase in tetracycline resistance genes can occur as a result of treatment processes (175). High prevalence has also been detected in direct hospital waste effluent (176), when on-site treatment occurs rather than connection to municipal waste treatment plants.

Understanding and mitigating the role of human development and anthropogenic climate change are crucial to reducing the spread of antibiotic resistance in the environment. Innovative developments in urban stormwater management such as green infrastructure (177) and new methods in monitoring and assessment (178) are aimed at reducing the human health risks of stormwater and sewage contamination events, including the risks associated with exposure to antibiotic resistant pathogens. Sewage spills are expected to increase with the effects of climate change (179), with greater frequency of extreme precipitation events (180, 181) and insufficient infrastructure, especially in vulnerable coastal communities such as Tampa Bay, FL (182), San Francisco, CA (183) and New York, NY (184). One study from The Netherlands used quantitative microbial risk assessment to determine that climate change is also expected to increase the risk of infection associated with exposure to pathogens downstream of wastewater treatment plants, due to reduced surface water flow rates, and subsequently reduced dilution factors (185).

*Animal agriculture*

Antibiotic resistant bacteria and antibiotic resistance genes are prevalent in swine, cattle, poultry and other animal husbandry industries (140, 186-188). The antibiotic resistance determinants targeted in agricultural ecosystem studies often depend on the type of husbandry and the types of animals being raised. Chapter 3 of this dissertation includes additional review
of the environmental prevalence of antibiotic resistance targets associated with animal agriculture.

**Vancomycin-Resistant Enterococci (VRE)**

*Enterococcus* spp. are aerotolerant, fermentative Gram-positive bacteria that can survive environmental stressors including considerable variability in nutrients, salinity and temperature (189). Enterococci are opportunistic pathogens but are found in the natural gut flora of healthy animals and used in regulatory standards for recreational water quality indicators to confirm the presence of fecal contamination (190), based on association between exposure and gastrointestinal illness(129, 191). *Enterococcus* spp. can persist in environmental reservoirs, such as water, vegetation and sediment, and are often attached to particles, especially in areas with high organic content (156, 192). Infections caused by pathogenic *Enterococcus* spp. can usually be treated with vancomycin, but incidence of resistance to vancomycin has been increasing. Almost all clinical enterococcal infections (95%) are caused by two species, *E. faecalis* and *E. faecium* (193). Vancomycin-resistant enterococci are the 3rd most common hospital-associated pathogens after methicillin resistant *Staphylococcus aureus* and *Clostridium difficile* (193).

Vancomycin is a glycopeptide antibiotic that inhibits cell wall cross-linking by binding to the sequence D-alanyl-D-alanine in the peptidoglycan of Gram-positive bacteria (194). Vancomycin resistance genes change peptidoglycan precursors by changing the amino acid chain sequence (to D-alanyl-D-lactate/serine) so that vancomycin does not readily bind to the bacteria (103, 195). Vancomycin is derived from the soil bacterium *Amycolatopsis orientalis*, from which it was first isolated in 1953. The drug was first introduced to healthcare markets in 1972 to treat infections caused by methicillin-resistant *Staphylococcus* spp., but the first cases of
resistance were observed in staphylococci 1979 (12). Approximately 66,000 cases of VRE occur annually in the United States (2). They are currently treated with linezolid and quinupristin/dalfopristin (196), although resistance to those drugs is now being reported (197). Vancomycin-resistant *Staphylococcus aureus* (VRSA) are also a grave and increasingly urgent concern to public health, with failures of last resort treatments. Cases of VRSA have been reported in at least eight different countries around the world, including at least 13 in the United States, though incidence of these infections is still relatively rare (198-200). Vancomycin-resistant enterococci have been detected in animal feces, drinking water, surface water, sewage, wastewater treatment plants and waste lagoons.

The glycopeptide class of antibiotics also includes another drug, avoparicin, which was used heavily in Europe in animal agriculture prior to its ban in the 1990’s. Use of avoparicin in Europe proved the direct evidence of animal to human transmission and connection in the antibiotic resistance framework, with genetic and evolutionary confirmation showing that avoparicin-resistant strains that proliferated in animal agriculture were also vancomycin-resistant, and caused infections in human populations (201). Many gene clusters or operons have been identified in association with vancomycin resistance, including accessory genes (*vanW*, *vanY*, *vanZ*) and resistance genes (*vanA, vanB, vanC, vanD*) (102, 202-204). Understanding gene functions and inherent versus acquired resistance helps to prioritize which genes and bacteria to target when studying VRE outside of hospitals.

The most prevalent VRE in clinical settings are *E. faecium* and *E. faecalis*, with high level resistance conferred by the *vanA* and *vanB* genes respectively, often at minimum inhibitory concentrations of >256 μg/mL. The *vanA* type *E. faecium* are effective targets in assessing

---

4Extensive review of prevalence of vancomycin-resistant enterococci in the environment is discussed in Chapters 2 and 4 of this dissertation.
mobility and the spread of resistance in the environment because of their clinical relevance and the association of \textit{vanA} with mobile plasmids (102). Many VRE have chromosomally encoded resistance to low levels (<32 µg/mL) of vancomycin, specifically \textit{Enterococcus gallinarum} and \textit{E. casseliflavus}/\textit{E. flavescens} (193). These species and their resistance genes (\textit{vanC}) are frequently detected in the environment, but do not pose risks to human health (205, 206). Mobility of vancomycin resistance genes is an important factor in the potential for uptake by pathogens and environmental bacteria in aquatic ecosystems, through incorporation of resistance genes by horizontal gene transfer, and transduction through bacteriophage (207).

Phylogenetic analysis (multi locus sequence typing) has identified a common, globally distributed, hospital-associated clade of VRE called CC-17 (clonal complex 17), which has been used as a reference to track the spread and evolutionary history of VRE outside of hospitals (208, 209). Detection of vancomycin-resistant \textit{E. faecium} of the hospital clonal lineage CC17 has been reported in wildlife including wild boar (210), swine (211), wolf (212), and fish (213) It has also been reported in food samples (214, 215). Vancomycin-resistant enterococci pose a major health risk in hospitals, and their ecology outside of clinical settings is an important area of study, including how environmental variability, genetic makeup of strains and molecular mechanisms of resistance can influence survival and persistence.

\textbf{Research Chapters: Goals of the Dissertation}

\textit{Chapter 2: Vancomycin-Resistant Enterococci in a Residential Sewage Spill}

\textbf{Hypothesis:} Culturable VRE will be detected for less time than the \textit{vanA} genes, and the \textit{vanA} genes will be detected in high concentrated sewage overflow events.

\textbf{Methodology:} Field sampling of sewage contamination events in Tampa Bay were conducted to test for VRE and \textit{vanA} genes and determine persistence over time.
**Hypothesis:** Microbial communities in aquatic environments are different immediately after a sewage spill and impacted by allochthonous, sewage-associated bacteria.

**Methodology:** Illumina sequencing was conducted to examine the changes in microbial community structure after a major sewage spill event.

*Chapter 3: Persistence of Antibiotic Resistance Genes in Poultry Litter Microcosms*

**Hypothesis:** Persistence of antibiotic resistance genes varies based on environmental matrix; genes persist longer in sediment than in water. Aquatic environments can act as reservoirs of antibiotic resistance genes.

**Methodology:** Microcosms were constructed to assess the persistence of clinically relevant antibiotic resistance genes associated with poultry litter in water and sediment of freshwater and marine environments.

*Chapter 4: Survival of Vancomycin-Resistant Enterococci in Ambient Waters*

**Hypothesis:** Larger plasmids are associated with a higher fitness cost and vancomycin-resistant enterococci with large plasmids have faster decay rates than those with no plasmid or a smaller plasmid.

**Methodology:** Microcosms were constructed to assess the role of plasmid size on the survival of vancomycin-resistant enterococci that were otherwise chromosomally identical.
References

3. FDA. 2016. Summary Report on Antimicrobials Sold or Distributed for Use in Food-Producing Animals. Department of Health and Human Services,


193. CDC. 2017. Vancomycin-resistant Enterococci (VRE) and the Clinical Laboratory.


CHAPTER 2: VANCOMYCIN-RESISTANT ENTEROCOCCI AND BACTERIAL COMMUNITY STRUCTURE FOLLOWIGN A SEWAGE SPILL INTO AN AQUATIC ENVIRONMENT

[This chapter has been published and is attached as Appendix B.]
CHAPTER 3: PERSISTENCE OF ANTIBIOTIC RESISTANCE GENES FROM POULTRY LITTER IN AQUATIC ENVIRONMENTS

Abstract

Runoff and waste from animal agricultural waste contributes to the spread of antibiotic resistance genes and antibiotic resistant bacteria in the environment. Empirical study of individual antibiotic resistance genes that are relevant to human health may improve assessments of the impacts of animal agriculture on the ecology of antibiotic resistance. Waste from animal agriculture, including the poultry industry, can impact aquatic environments and public health by introducing antibiotic resistance genes and their associated pathogens. Microcosms were constructed to assess the persistence of antibiotic resistance genes associated with poultry litter (waste mixed with bedding) in water and sediment from fresh and marine waters. Five antibiotic resistance genes (ampC, ermB, qnrS, tetB, vanA) were assessed in initial poultry litter, water and sediment by PCR. Three antibiotic resistance genes that were detected by conventional PCR (ampC, ermB, tetB) were quantified using qPCR over a seven-day microcosm study. Decay rates for all resistance genes were similar in water, with decreasing concentration over 7 days, but concentrations increased in sediments for ermB and tetB resistance genes. The three resistance genes remained in concentrations detectable by qPCR for at least one week, in both freshwater and marine environments. Soils or sediments in agricultural ecosystems impacted by animal
waste may act as environmental reservoirs in which antibiotic resistance genes and antibiotic-resistant bacteria can persist and increase.

**Background**

Antibiotic resistance genes are considered contaminants in aquatic ecosystems and can be released into the environment through wastewater effluent and animal agricultural waste (1-3). The use of antibiotics in animal agriculture has been linked to the spread of antibiotic resistance in human health and clinical infection (4-8). Assessing the sources, persistence and fate of antibiotic resistance genes in aquatic ecosystems requires specific targeting of genes that are relevant to human health and the agricultural industry of concern. Based on commonly targeted genes and antibiotics used in poultry industry, five antibiotic resistance genes were assessed in microcosms with poultry litter (the mix of animal waste and bedding) to determine prevalence and persistence in soil and water of freshwater and marine environments.

Antibiotics are used in the poultry industry to prevent infection and promote growth, in addition to their original therapeutic use as treatment for bacterial infections (9). The genes conferring resistance to beta-lactams (*ampC*), macrolides (*ermB*), tetracyclines (*tetB*) were targeted in this study based on their use in poultry farming in the United States and relevance to human health (10, 11). Genes conferring resistance to quinolone antibiotics have not been detected in animal agriculture ecosystems frequently, but more recent reports of detection in poultry waste prompted the inclusion of the plasmid-mediated *qnrS* gene in this study (12, 13). The vancomycin resistance gene *vanA* was also assessed because of its historical relevance with animal agriculture in Europe, though these classes of antibiotics are not used in the poultry industry in the United States (14).
Antibiotic resistance genes are often associated with zoonotic and human pathogens.
Bacteria such as *Escherichia coli*, *Campylobacter* spp. and *Salmonella* spp. are known to transfer through poultry waste and meat products, sometimes causing disease outbreaks (15). In fact, the most common food associated foodborne outbreaks in the United States are linked to poultry-based product (16). While this study does not assess the food products or their consumption, the relevant antibiotic resistance genes and pathogens are often excreted in waste; between 25% and 75% of compound is not fully metabolized and can reach adjacent environments through runoff or when applied as fertilizer (17, 18). The waste from animal agriculture industries (livestock and poultry) amounts to more than 180 million tons annually in the United States (19).

Antibiotic resistance genes can spread through horizontal gene transfer once deposited into environmental matrices such as surface water and sediments (20, 21). Even if pathogens are not resistant to antibiotics when they enter the environment, they can acquire resistance through the lateral transfer of antibiotic resistance genes, or horizontal gene transfer. Horizontal gene transfer has been studied in culture experiments but actual rates of transfer in environmental settings are difficult to quantify because of the relative infrequency of these events (22).

The connection between antibiotic use in animal agriculture and antibiotic resistance in human pathogens has been studied extensively (23, 24), most distinctly in Europe with the use of avoparcin, a cognate of vancomycin in the same glycopeptide class, in animal farming and correlated with an increase of human infections caused by vancomycin resistant enterococci (25). Increased reports of these zoonotic transmissions triggered a ban of antibiotics used for growth promotion in Europe, Taiwan and other areas, which successfully reduced prevalence of vancomycin resistant enterococci (26-28).
Few studies have performed quantitative analyses or addressed the fate and persistence of antibiotic resistance genes in aquatic ecosystems impacted by poultry waste. Most research has addressed the detection of antibiotic resistance genes in waste lagoons or manure, and phenotypic resistance has been detected in bacterial isolates from poultry litter (29-33). Antibiotic resistance genes can persist for months in agricultural soils where manure is applied as land fertilizer, though data on smaller timescales are not widely reported (34-36). This study aimed to assess the fate of antibiotic resistance genes associated with poultry litter in aquatic ecosystems, as areas receiving animal waste and runoff, by examining surface waters and associated sediments in microcosms.

Resistance to beta-lactam antibiotics is often detected in environmental matrices by targeting the ampC genes, including the plasmid-mediated bla\textsubscript{CMY-2} gene (37). Beta-lactam antibiotics include penicillins and 1\textsuperscript{st}, 2\textsuperscript{nd} and 3\textsuperscript{rd} generation cephalosporins and are used to treat infections caused by Gram-negative pathogens (38). Beta-lactam classes of antibiotics have a mode of action whereby penicillin-binding proteins are inhibited, thus preventing cell wall cross-linking (39). Bacteria that produce the extended spectrum beta-lactamase (ESBL) enzymes are resistant to the beta-lactam drugs (40) and therefore a growing concern in human and animal health (41, 42). The ampC resistance determinants have been detected in the poultry chain (43, 44), Salmonella spp. and E. coli isolates from cow, pig and human samples (45, 46), in swine and ground pork (47), and also in wild animals (48, 49). Persistence of ampC has been quantified in digestive tracts of chickens and can transfer in vivo to pathogens E. coli and Klebsiella pneumoniae (50). The dominant pathogens of concern that may carry the ampC genes are Salmonella spp. and E. coli (51).
Macrolides are antibiotic class that inhibit protein synthesis through various pathways, binding to the large ribosomal subunit to disrupt synthesis of proteins (52, 53) Erythromycin is a common drug in the macrolide class, and resistance mechanisms have been identified as target enzymes, of which more than 40 \textit{erm} genes have been identified (54-56). \textit{ermB} genes are one of the four major classes of \textit{erm} resistance determinants that are associated with pathogens, in addition to \textit{ermA}, \textit{ermC} and \textit{ermF} (56). \textit{ermB} genes have been detected in swine (57, 58), farm soil (59) cow manure (60), poultry cecum (61, 62) and poultry litter (63, 64). \textit{ermB} genes have also been detected in environmental sources in the vicinity of animal agricultural production facilities (65). Bacterial pathogens most likely to be resistant to macrolides and harbor \textit{ermB} genes include \textit{Staphylococcus aureus}, \textit{Enterococcus faecium} and \textit{Escherichia coli} (66-71).

Tetracycline antibiotics disrupt protein synthesis by binding to the attachment of aminoacyl-tRNA to ribosomal acceptor (A) site (72). Tetracycline resistance genes were targeted because of use of tetracyclines in animal feed and water in the poultry industry (73, 74). Dozens of tetracycline resistance genes have been identified, and the \textit{tetB} gene was chosen based on its historical prevalence and its use in recent studies of poultry litter (55, 75-78). Tetracycline antibiotics are used widely to treat a variety of infections and parasites, including use in malaria prevention as doxycycline and treatment for syphilis and methicillin-resistant \textit{Staphylococcus aureus} or MRSA (72).

Quinolone drug classes target DNA gyrase and topoisomerase IV, essential enzymes to nucleic acid processes in bacterial reproduction (79, 80). Quinolone resistance was assessed through the \textit{qnrS} gene, a plasmid-mediated gene of Qnr proteins that protect the target enzymes (81, 82). Although the \textit{qnrS} genes have not been targeted or detected frequently in poultry waste, quinolones are common drugs for human use including Ciprofloxacin and the inclusion in
this study was based on the mobility of these genes and the potential implications for human health. The association with

Vancomycin is a glycopeptide drug that inhibits cell wall synthesis. Glycopeptides are not used in animal agriculture in the United States but were used in European poultry farming and linked to the occurrence of glycopeptide resistance in clinical infections of human pathogens (26, 83-85). Transfer of the vanA gene from animal to human in vivo has been described (86), but vancomycin resistant enterococci with vanA have only been detected on animal farming operations in the United States in one study (14). Vancomycin resistant enterococci, specifically E. faecium and E. faecalis, are the third most common hospital-acquired infection in the United States\(^1\).

Background environmental levels of antibiotic-resistant bacteria and antibiotic resistance genes in aquatic ecosystems (including surface water and sediments) are rarely measured, and longitudinal studies that monitor antibiotic resistance in the environment are rare. Targeted empirical studies can assess dynamics of persistence more robustly in practical laboratory settings. Few studies have investigated the persistence of antibiotic resistance genes in receiving waters through simulated ecosystems (87), but none have used water and soil matrices from different sources. In these microcosm studies, antibiotic resistance genes were inoculated through mixing poultry litter with sediment from freshwater and marine sources in Tampa Bay, Florida, to assess their persistence in both water and sediment. Persistence of fecal indicators and microbial source tracking markers was also assessed to gain perspective on monitoring antibiotic resistance in aquatic environments.

---

\(^1\) For more on vancomycin and vancomycin-resistant enterococci, see Chapter 2 and Chapter 4 of this dissertation.
Note: The results presented here are part of a larger manuscript in progress that includes an additional microcosm experiment for seasonal comparisons, bacterial quantifications of *Salmonella* spp., *E. coli*, *Enterococcus* spp. and a microbial source tracking marker for poultry (*Brevibacterium* sp. LA35). Those data will also be compared to a previously published study conducting similar experiments, showing seasonal variability (88).

**Materials and Methods**

**Microcosm Set-up**

Microcosms were constructed in triplicate using 2-liter glass beakers as previously described and monitored for seven days (88). Soil from a freshwater environment (Hillsborough River, Tampa, FL) or a marine environment (Fort DeSoto Beach, St. Petersburg, FL) was mixed with 10 grams poultry litter from a conventional poultry farming operation in West Virginia and placed in a 1-inch layer at the bottoms of the beakers. Water from corresponding locations was added in 1-liter volumes. Hobo loggers were kept at the microcosm sites (greenhouse of the USF Botanical Garden, Tampa, FL) for the entire length of the experiments to monitor temperature and light intensity. Microcosms were protected from physical intrusion using saran wrap covers and chicken wire enclosure, but vulnerable to various environmental stressors including temperature and sunlight.

**Sampling Procedures**

Samples were collected as previously described (88). Briefly, 20 mL of water and 5 g of soil were collected separately from each microcosm in 5-gram using sterile 50-mL tubes. Water was collected mid-column, without disturbing the sediment layer. Sediment layer samples were collected after the water samples were collected to prevent cross-contamination. Samples were collected daily on Days 1-4 and on the last day of the experiment, Day 7.
Detection of Antibiotic Resistance Genes by PCR and qPCR

DNA was extracted using PowerWater and PowerSoil DNA Extraction Kits (MoBio/Qiagen, Carlsbad, CA). Poultry litter was tested for all five antibiotic resistance genes using conventional PCR (Table 1). Substrates (water and sediment) were tested for presence of antibiotic resistance genes before adding the poultry litter to test for natural occurrence of targets. Antibiotic resistance genes that were detected by conventional PCR were quantified by SYBR or TaqMan qPCR in DNA extracted from water and sediment in each microcosm for each of the sampling dates (Days 1, 2, 3, 4, 7). Table 1 provides the primers for each antibiotic resistance gene target (and probe for tetB where TaqMan qPCR was used). Each SYBR assay was performed with GoTaq qPCR MasterMix (ProMega, Fitchburg, WI) and the TaqMan assay for tetB was performed using TaqMan Environmental MasterMix 2.0 (Applied Biosystems, Waltham, MA). The assay for beta-lactam resistance (ampC) was developed based on the GenBank sequence for the plasmid-mediated CMY2 (blaCMY-2) enzymes (Accession NC_0114114.1). All positive controls for PCR and standard curves for qPCR were synthetic plasmids constructed from known sequences as cited in Table 1 (IDT, Coralville, IA).

Analysis

Data were analyzed based on binary logistic regression with PCR data (SPSS). Log10 reductions to assess decay were calculated using previously described formulae and methods (89). Briefly, decay was calculated based on the ratio of the concentrations at Day 7 and Day 1. Data for log10 reductions were compared using ordinary 2-way ANOVA analysis, comparing different matrices and different environments (freshwater or marine). One-way ANOVA tests were used in GraphPad Prism (Version 6.07, GraphPad Software Inc., La Jolla, CA) to determine differences in decay among individual antibiotic resistance genes within matrices.
Results

Detection by PCR

Three antibiotic resistance genes, \textit{ampC}, \textit{ermB} and \textit{tetB}, were detected in poultry litter by conventional PCR but \textit{qnrS} and \textit{vanA} were not detected. None of the antibiotic resistance genes were detected by conventional PCR in sediment or water from fresh or marine sources prior to inoculation with poultry litter. DNA extractions from each of the sampling timepoints (Day 1, 2, 3, 4, 7) were tested for each of the antibiotic resistance genes that were detected in initial poultry litter (\textit{ampC}, \textit{ermB}, \textit{tetB}). Persistence of antibiotic resistance genes varied across genes and environmental matrices, after poultry litter was mixed with sediment at the start of the experiment (Table 2). \textit{ampC} genes persisted for the entire experiment in river sediment but only for 3 days in river water, and 4 days in marine water and marine sediment. \textit{ermB} genes persisted for 7 days in river water but were not detectable after 3 days in river sediment, marine water and marine sediment. \textit{tetB} genes persisted for 7 days in all matrices. Correlations using binary logistic regression determined no significant relationship between the antibiotic resistance genes. Correlations were also assessed using data for bacterial species and microbial source tracking markers, not reported here.

Measurement by qPCR

Antibiotic resistance genes that were detected by PCR (\textit{ampC}, \textit{ermB} and \textit{tetB}) were quantified by qPCR in sediment and water of each microcosm for each timepoint (Day 1, 2, 3, 4, 7). In water samples, high concentrations of all antibiotic resistance genes on Day 1 ($10^6$-$10^8$ copies/100mL) reduced by 2-4 logs over 7 days (Figure 2). Concentrations of \textit{ampC} and \textit{tetB} increased in both marine and freshwater sediment, contributing to the significant effect of matrix for each gene ($P=0.002$ and $P=0.0002$, respectively; one-way ANOVA; Figure 1 and Figure 2).
ermB concentrations decreased over time in marine sediment, though not significantly different from other matrices (Figure 2). An interaction between resistance gene target and matrix was observed \((P=0.008)\), and a significant effect of environmental matrix was detected \((P<0.0001)\).

Volumes of DNA extracts were limited and initial environmental samples were depleted before qPCR could be assessed for all antibiotic resistance genes in the sediment and water used in the microcosms. Poultry litter samples were successfully tested by qPCR for the three antibiotic resistance genes \(ampC\), \(tetB\) and \(ermB\). Quantities were between \(10^6\)-\(10^9\) copies/100g, confirming the poultry litter as the primary source of antibiotic resistance genes in the microcosms.

Correlations between antibiotic resistance genes, fecal indicators \(Enterococcus\) spp., bacterial pathogens \(Salmonella\) spp. and a microbial source tracking markers for poultry \(Brevibacterium\) sp. LA35 were also analyzed as part of a larger study and not presented in detail here. This microcosm was completed in winter, while a previous microcosm was completed in spring, and additional analyses will include assessment of seasonal variability in persistence. In general, antibiotic resistance genes correlated with each other and bacteria in water using correlations with Pearson’s \(r\). All relationships were significant in freshwater and marine water matrices \((P<0.05)\) except the \(ermB\) and \(Enterococcus\) spp. in freshwater \((P=0.056)\). However there was much more variability in correlations statistics for sediment matrices. These analyses are in agreement with previous analyses of indicators and pathogens correlated to the LA35 poultry fecal marker (88). Further comprehensive analyses are in progress to incorporate all data into one manuscript.
Figure 1. Concentrations (log_{10} gene copies per 100mL or 100g) in microcosms over time in each matrix. Points represent mean concentration and error bars are the range of triplicate microcosms.
Figure 2. Decay of antibiotic resistance genes *ampC, ermB, tetB* calculated as log_{10} reduction (the difference in Day 7 and Day 1 concentrations) for each environmental matrix. Values > 0 denote increased gene concentration over time.
Table 1. PCR conditions for PCR and qPCR assays targeting antibiotic resistance genes.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers (5' → 3') and probe</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ampC</td>
<td>F: CCCGATGACGTTAGGGATAAAG R: CTAACTCCAGCATTGGTCTGT</td>
<td>This study</td>
</tr>
<tr>
<td>ermB</td>
<td>F: GATACCGTTTACGAAATTGG R: GCACACTCAAGTCTCGATT</td>
<td>(3)</td>
</tr>
<tr>
<td>qnrS</td>
<td>F: ACGACATTCGTCAAACCTGCAA R: GCCTACAGGCTGCCAATT</td>
<td>(90)</td>
</tr>
<tr>
<td>tetB</td>
<td>F: AGGCGCATCGCTGGATT R: CAGCATCCAAAGCGCAGTT 6FAM-CTTATTGCTGGCTTTTTT (MGB Taqman probe)</td>
<td>(91)</td>
</tr>
<tr>
<td>vanA</td>
<td>F: CTGTGAGGTCGGTTGCGCG R: TTTGGTCCACCTCGCCA</td>
<td>(92)</td>
</tr>
</tbody>
</table>

Table 2. Persistence of antibiotic resistance genes assessed by conventional PCR.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Last day of detection (of 7 day experiment)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ermB</td>
</tr>
<tr>
<td>Marine water</td>
<td>3</td>
</tr>
<tr>
<td>Marine sediment</td>
<td>3</td>
</tr>
<tr>
<td>River water</td>
<td>7</td>
</tr>
<tr>
<td>River sediment</td>
<td>3</td>
</tr>
</tbody>
</table>
Discussion

Poultry litter contains antibiotic resistance genes that can be released into and persist in the environment. The antibiotic resistance genes in this study are relevant to human health and can persist differently in different environmental matrices. Previous studies have detected the antibiotic resistance genes and antibiotic resistant bacteria in animal waste and in surface waters or sediments receiving animal waste or manure fertilizer (93-95). Poultry litter and aquatic environments adjacent to poultry farming operations have not been extensively tested for these resistance genes to directly compare their persistence. The concentrations of \textit{ermB}, \textit{ampC} and \textit{tetB} in the initial poultry litter inoculant show that these genetic elements are present in high levels, beyond phenotypic bacterial resistance that is commonly measured. This is particularly relevant for mobile resistance elements associated with plasmids, such as \textit{ampC}. Decay in water and sediment over time shows that when released into the environment via poultry litter waste, these genes can persist for at least one week and potentially increase in sediment.

Persistence of the relevant genes is directly related to the survival dynamics of bacteria that harbor antibiotic resistance genes. Studies in natural environments have shown that survival of bacteria that contain antibiotic resistance genes (\textit{Enterococcus} spp. and \textit{E. coli}), are impacted by ambient sunlight, nutrient levels and biotic factors (competition, predation, viral lysis) (89, 96, 97). While these factors were not directly assessed as mechanisms of decay of antibiotic resistance genes, the variability between matrices and between freshwater and marine sources underscore the previously determined role of biotic and abiotic factors in bacterial survival.

While antibiotic resistance genes persisted in all matrices, concentrations of \textit{ampC} and \textit{tetB} increased in sediment samples. These increases are likely linked to the survival or replication of the bacteria that harbor those genes. Variability in decay of antibiotic resistance genes in
different environmental matrices creates complexities in assessing their sources and fate in aquatic ecosystems. A recent study using microcosms to assess natural attenuation of antibiotic resistance genes after application of animal manure found that concentrations of \( \text{ermB} \) and \( \text{tetB} \) reduced more in manure-pond microcosms than manure-soil microcosms (98). The results from previous studies are in agreement with the attenuation and increase in concentration in sediment samples in this study.

\( \text{ermB} \) can persist long-term in areas impacted by animal agriculture, detected annually from waste lagoons, for weeks in simulated storage experiments and beyond 175 days in cattle feces (99-101). \( \text{ermB} \) has also been detected in direct receiving drainage of fields that use swine manure as fertilizer at \( 10^4 \text{ to } 10^6 \) gene copies/100mL, similar to concentrations of \( \text{ermB} \) in water samples from microcosms after sediment was mixed with poultry litter in this study (102). Concentrations of \( \text{ermB} \) were detected and decreased in all matrices, as opposed to the increase in concentration observed in sediment for \( \text{ampC} \) and \( \text{tetB} \). Other studies focus on manure and waste and have not assessed the persistence of \( \text{ermB} \) in water or sediment, so there are no opportunities for direct comparison. Concentrations of \( \text{ermB} \) over time confirm the decline of these genes in environmental water and sediments but also their persistence in all matrices for at least one week.

Tetracycline resistance genes are prevalent in environments impacted by animal agricultural waste but \( \text{tetB} \) was not detected by conventional PCR in the natural environmental substrates used for the microcosms. However, this is likely due to the high limit of detection for the conventional PCR assay. Tetracycline resistance genes have also been found in relatively pristine aquatic environments and in wild animals (103, 104) and are ubiquitous in animal agricultural waste, including detection in human pathogens and in soils where manure is applied.
as fertilizer (45, 105, 106). Concentrations of tetB in composted chicken manure, approximately $10^7$ gene copies per gram, were similar to concentrations in poultry litter in this study (106).

Over the seven-day study, the tetB gene decreased in concentration in water but increased in sediment. However, it is inappropriate to interpret that this gene represents all tetracycline resistance genes; one mesocosm study found different survival patterns among six tetracycline resistance genes in wastewater lagoons, and the tetW gene was most persistent (107). Further studies that determine the fates of individual resistance genes in these types of ecologically relevant experiments can be used to prioritize genes and pathogens for monitoring and surveillance.

Concentrations of the beta-lactam resistance gene ampC also increased in sediment while they decreased in water, similar to the decay of the tetB genes. Persistence of ampC has been assessed in medical contexts (108, 109) but not as frequently in non-clinical, environmental contexts. Recently, ESBL-producing E. coli and their ampC genes have been detected on fresh produce and in an urban river in India also support the results of this study that the ampC genes and the bacteria that harbor them can persist outside nutrient-rich environments (110, 111).

Different results in detection between conventional PCR and qPCR support the need to consider detection limits of assays when choosing what methods to use in different systems. The signal was lost on Day 4 in some matrices for ampC and ermB using conventional PCR, but gene copies were detectable throughout the entire experiment using qPCR. qPCR is a more sensitive method for detecting antibiotic resistance genes in aquatic environments.

Advanced approaches that assess broad resistomes have revealed patterns based on antibiotic use in animal agriculture, with human gut microbiomes showing more resistance to antibiotics used in animal agriculture as opposed to antibiotics not used in animal agriculture
Poultry sources can influence human resistomes, especially in rural areas where human contact with the animals is frequent and housing is adjacent, which has been shown as an avenue for the transfer of antibiotic resistance genes (113).

The quantities of antibiotics used in animal agriculture have increased dramatically in the recent decades, and legislative efforts to limit their use have not been successful in the United States (114). This is exemplified by the failures of many congressional bills including The Preservation of Antibiotics for Human Diseases Act of 1999, The Strategies to Address Antimicrobial Resistance (STAAR) Act of 2010, The Delivering Antimicrobial Transparency in Animals Act of 2012, and The Preservation of Antibiotics for Medical Treatment Act of 2013, among others (115-117). However, a recent change in federal policy may help to reduce non-therapeutic use of antibiotics in animal agriculture, through a veterinary feed directive with specific guidelines regarding antibiotic prescription (118).

This study examined the persistence of three antibiotic resistance genes of relevance to human and animal health in ecologically relevant microcosms. Their detection, persistence and variable survival in different environmental matrices provides insight into the ecology of antibiotic resistance. Poultry litter contains clinically relevant antibiotic resistance genes that can persist in both water and sediment, with the potential for concentrations to increase in sediments. Further studies are necessary to identify the specific mechanisms of survival and directly connect the resistance genes to the bacterial pathogens that harbor them. These results contribute to understanding dynamics of antibiotic resistance in aquatic environments by showing differential persistence of antibiotic resistance genes in water and sediment. Monitoring, management and mitigation strategies can be designed to more effectively address contamination of clinically
relevant genes based on these temporal data, conducted in natural environments with poultry litter.

References

encoded AmpC–producing Escherichia coli from food and farm animals, Sweden. Emerging infectious diseases 22:634.


68. Farrow KA, Lyras D, Rood JI. 2000. The macrolide-lincosamide-streptogramin B resistance determinant from Clostridium difficile 630 contains two erm (B) genes. Antimicrobial agents and chemotherapy 44:411-413.


CHAPTER 4: NUTRIENT-DEPENDENT EFFECT OF PLASMIDS CONFERRING HIGH-LEVEL VANCOMYCIN RESISTANCE ON PERSISTENCE OF ENTEROCOCCUS FAECIUM IN AMBIENT WATERS

Abstract

Vancomycin resistant enterococci (VRE) cause 20,000 hospital-acquired infections annually in the United States but are not well studied in environmental contexts. Recent findings of VRE in water following a sewage spill suggest that the spread of vancomycin resistance can occur in environmental settings, which could be facilitated by mobile genetic elements such as plasmids and transposons. Maintenance of antibiotic resistance genes and proteins may, however, present a cost of fitness in the absence of selective pressure. Nutrient levels in receiving environments may also affect survival when organisms enter aquatic ecosystems through sewage spills. To test the hypotheses that nutrients and/or plasmids conferring vancomycin resistance can affect Enterococcus faecium survival in ambient waters, survival of strains that differed only by the plasmid they carried was assessed in river water under natural and augmented nutrient levels. Under natural nutrient levels, plasmid size correlated directly with decay rate (log$_{10}$ reduction), but under augmented nutrient conditions plasmid presence and size had no effect on decay rate. The vancomycin-resistant strain with the largest plasmid (200 kb) decayed significantly more rapidly than the susceptible parent strain, which had no
plasmid, while strains with smaller plasmids did not have significantly different decay compared to the parent strain. This work is among the first to show that plasmids conferring antibiotic resistance affect fitness of Enterococcus species in secondary habitats such as surface water.

Background

Vancomycin resistant enterococci (VRE) cause 20,000 hospital-acquired infections and 1,300 deaths annually in the United States, and are categorized as a serious public health threat (1). Nosocomial and community-acquired infections caused by VRE are on the rise (2-6), and VRE and the mobile, high-level resistance gene vanA have been detected in aquatic environments, wildlife feces and human sewage (7-14). VRE infections are commonly monitored through surveillance programs in healthcare systems and often studied in these contexts, but conditions that contribute to VRE survival in environmental reservoirs are poorly understood.

Enterococcus spp. can carry mobile genetic elements that confer antibiotic resistance (15) and nine different gene clusters conferring vancomycin resistance have been identified (16) but not all are relevant from a human health perspective. For example, the vanC gene confers intrinsic, low-level resistance (2-32 μg•mL⁻¹) and is commonly detected in environmental enterococci, including E. gallinarum (17). Thus, detection of vanC in the aquatic environment is not an effective indicator of risk to human health. The most clinically relevant VRE are the high-level resistant E. faecium carrying the vanA gene (18), with a minimum inhibitory concentration of ≥ 32 μg•mL⁻¹ vancomycin (18, 19). The vanA operon is generally located on a plasmid, including transposons or gene cassettes that facilitate mobility (20-22). The potential for spread of
high-level resistance to other pathogens such as *Staphylococcus aureus*, and/or environmental bacteria, make the environmental persistence of bacteria bearing genetic elements carrying *vanA* a relevant public health concern (23, 24).

Many studies have examined the role of bacterial plasmids in virulence and biological fitness using competitive culture or mouse model experiments (25-27). Maintenance of plasmids that confer antibiotic resistance can present a fitness cost and large plasmids are usually found in low copy numbers (28-38). The cost of resistance can be too great to sustain in the absence of selective pressure, and some bacteria lose plasmids when under stress to reduce metabolic expense (34). However, laboratory fitness experiments with VRE in culture have shown that the cost of plasmid-associated resistance depends on the host and the plasmid (35) and that plasmids can be maintained in the absence of selective pressure (39, 40) and in starvation states (32).

Nutrient availability is a key factor to consider when studying microbial interactions and survival of bacteria in aquatic environments (41-43). *Enterococcus* spp. are fermentative, aerotolerant anaerobes, and have broad metabolic capabilities, allowing them to compete in diverse environments (43, 44). Organic carbon levels in aquatic ecosystems influence survival of fecal-derived bacteria including enterococci (37, 42, 45-49). In this study, carbon sources used for augmented nutrient conditions in microcosms included common carbohydrates glucose, pyruvate and acetate. The aquatic environment provides relatively oligotrophic conditions compared to culture media or intestinal environments (50) and the interactions between resistance plasmids and nutrients need further study in oligotrophic settings.
Few studies have investigated how nutrients interact with maintenance of antibiotic resistance plasmids outside of nutrient-rich culture experiments. In this study, chromosomally identical strains of *E. faecium* with vancomycin resistance plasmids of differing sizes and genetic makeup were compared in microcosms containing filtered river water maintained in an outdoor environment. Nutrient levels were natural, or amended with trace vitamins, minerals and organic carbon sources glucose, pyruvate and acetate. Investigating bacterial persistence associated with plasmids and environmental nutrient levels contribute to understanding antibiotic resistance in a dynamic, ecologically relevant context.

**Materials and Methods**

*Enterococcus faecium strains*

Four *E. faecium* strains were obtained from Dr. Guido Werner (Robert Koch Institute, Wernigerode, Germany). In a previous study, clinically-derived strains carrying plasmids with the vanA gene were filter-mated with the vancomycin-sensitive American Type Culture Collection (ATCC) strain 64/3 (40). Each VRE strain in Table 1 represents a transconjugant of 64/3 (recipient strain) with the *E. faecium* plasmid donors, with plasmid sizes ranging from 50-200kb. Previous testing showed stable maintenance of plasmids on media without antibiotics for approximately 100 generations (40). Additional testing confirmed that strains maintained plasmids and survived for up to two weeks in phosphate-buffered water and filtered river water in microcosms maintained in the laboratory. Three transconjugant strains representing a range of plasmid sizes and characteristics were chosen for comparison of survival with the parent strain 64/3.
**Microcosm Set-up**

Water was collected from the Hillsborough River in Tampa, FL (28.069992, 82.377558) on March 31, 2017 in sterile, 50 L carboys. The water was filtered on that date using dialysis filters (REXEEED-25S, Asahi Kasei) to remove particles and microorganisms. Filtered water was tested for endogenous enterococci and VRE on mEI agar and mEI agar amended with 32 μg•mL⁻¹ vancomycin to confirm absence of culturable enterococci prior to the start of the experiment (51).

One liter of filtered river water was placed in each of 40 1.5 or 2 Liter beakers (5 beakers for each of 4 strains in both natural and augmented nutrient conditions) and covered with plastic wrap to allow light penetration and prevent contamination. A 1000-gallon capacity tank functioned as a water bath to modulate temperature fluctuations in the greenhouse at the USF Botanical Gardens. The greenhouse is protected from rainfall and covered to prevent animal intrusion, but is exposed to UV light and susceptible to environmental temperature changes. Microcosms were secured to shelves and partially submerged in the tank three days prior to the start of the experiment for acclimation time.

Carbohydrate sources were added as nutritional supplements in augmented nutrient conditions and compared to a control treatment with no nutrients added. Nutrient additions were as follows for the 1X treatment: hydrated sodium salts of pyruvate (8.3 mg•L), acetate (6.2 mg•L) and glucose (18 mg•L) for final concentrations of 0.0083, 0.0062 and 0.018 mg•L, respectively; pre-mixed additives for trace vitamins and minerals were added in 1 mL volumes of each mixture (ATCC, Manassas, VA) (45). Two HOBO data loggers (H08-004-02, Onset Computer Corporation, Bourne, MA) were placed in identical beakers with the same source water inside the water bath, where
measurements for temperature, humidity and light intensity were logged hourly over eight days. Light wavelength and intensity were recorded on each sampling date using a spectroradiometer (Model ILT950, International Light Technologies, Peabody, MA) at the time of collection.

Overnight cultures of each E. faecium strain were prepared in 10 mL brain heart infusion broth (Becton, Dickinson and Company, Sparks, MD, USA) using isolated colonies obtained from frozen stock cultures of VRE transconjugants E0292, H182, H74, and the parent strain, vancomycin sensitive 64/3. Cultures were incubated for 12 hours at 37°C, then transferred to a sterile 15 mL tube and centrifuged at 5000 rpm for 3 minutes. Supernatant broth was removed and 6 mL of sterile phosphate-buffered solution was added to each tube and vortexed. Individual strains were added to each of five replicate beakers as 1 mL of the vortexed overnight culture (approximately $1 \times 10^8$ cells $\cdot$ mL$^{-1}$ based on inoculants quantified on mEI agar) in both control and 1X nutrient treatments.

Sample Collection and Processing

Water samples were collected at the initial inoculation time (Day 0), and on Day 2, Day 4 and Day 7. Samples were collected from each microcosm (n=40) in 10 mL volumes using sterile pipets. Samples were transported on ice to the laboratory in sterile 15 mL centrifuge tubes, held on ice, and processed within 6 hours. Water samples were processed by membrane filtration for enterococci in duplicate, on both mEI and on mEI + 32 $\mu$g$\cdot$mL$^{-1}$ vancomycin, except for the non-resistant strain 64/3, which was only processed on unamended mEI (51). Dilutions were established to ensure a countable number of colonies (20-100 per plate). Estimates for dilution series were based on preliminary studies and earlier sampling results when processing samples at later time
points. VRE strains were cultured on both mEI and mEI with vancomycin on Day 0 and Day 4. Data from Day 7 were excluded from analyses because a majority of strains were undetectable by that date (<1 CFU •100mL⁻¹).

Data analysis

Culturable enterococci concentrations were recorded as CFU•mL⁻¹ and log₁₀ reduction was calculated for each replicate, based on the final concentration at Day 4 and the initial concentration at Day 0, using an equation as previously described (52). Briefly, the log reduction represents the ratio of log-transformed concentrations at Day 4 and Day 0. Day 4 was chosen based on preliminary studies to assess the most appropriate time point for capturing the decay rate before enterococci reached undetectable levels. GraphPad Prism was used to perform 2-way ANOVA with Sidak’s multiple comparison and linear correlation assuming Gaussian distribution with Pearson’s r (Version 6.07, GraphPad Software Inc., La Jolla, CA). Data from Day 7 were not included in analyses because a majority of strains were undetectable.

Results

Baseline nutrient concentrations of ammonium, nitrate and phosphorus in filtered water (0.165, 0.536 and 0.047 ppm, respectively) were consistent with Florida water quality guidelines and measurements from similar freshwater systems in the region (53). Plasmids in each transconjugant strain differed in their sizes and other characteristics as described previously (40) and are summarized in Table 1. Plasmid sizes ranged from 50kb to 200kb and were characterized by different genetic content, including toxin-antitoxin systems and replicon typing. Each transconjugant contained a plasmid from a clinical isolate, one from a hospital in the United States (E0292) and two from hospitals
in Portugal (H74 and H182) (Table 1). No significant differences in *E. faecium* concentrations on mEI vs. mEI+vancomycin were detected in natural or augmented nutrient conditions (2-way ANOVA; *P*=0.66 for and *P*=0.73, respectively), confirming that plasmids were maintained throughout the experiment.

Overnight cultures of transconjugants and the parent strain were quantified before dilution and inoculation into microcosms and were highly similar, with a mean of 5.27 x 10^8 CFU•mL^-1. Initial concentrations of the transconjugant strains in microcosms were more variable as measured from sampling at Day 0, with a mean of 4.4 x 10^7 CFU•100mL^-1. All strains declined to between 10^1-10^4 CFU•100mL^-1 after four days (Figure 1). Nutrients did not have a significant effect on log_{10} reduction (2-way ANOVA; *P*=0.3131; Figure 2) but there was a significant effect of strain (*P*=0.0064). Transconjugant E0292 experienced significantly greater log_{10} reduction than the parent strain, but only under natural nutrient conditions (Figure 2; *P*=0.022), with no added organic carbon sources.

Log_{10} reduction of the *E. faecium* strains was directly correlated with plasmid size in the natural nutrient treatment (Figure 3A; *P*=0.0207, r=0.5129), but not in the augmented nutrient treatment (Figure 3B; *P*=0.3998, r=0.1992). Interestingly, log_{10} reduction of the parent strain was not different between nutrient-augmented and natural nutrient treatments (*P*=0.3428), while log_{10} reduction of E0292 was significantly greater under natural conditions compared to nutrient-augmented conditions (*P*=0.0469). An interaction between strain and nutrient level was determined (Figure 2; 2-way ANOVA, *P*=0.0433).
Table 1. *E. faecium* strain origin and plasmid characteristics, adapted from Werner et al. 2011.

<table>
<thead>
<tr>
<th><em>E. faecium</em> strain</th>
<th>Origin</th>
<th>Country of origin</th>
<th>Year</th>
<th>Plasmid characteristics</th>
<th>Traits/mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>64/3</td>
<td>ATCC</td>
<td></td>
<td></td>
<td>None; recipient strain</td>
<td>N/A</td>
</tr>
<tr>
<td>H182</td>
<td>hepatic fluid</td>
<td>Portugal</td>
<td>2002</td>
<td>92 kb vanA plasmid (rep-pRUM)</td>
<td>MDR gene cassette (Rosvoll et al. 2010), inhibit growth (Grady and Hayes 2003)</td>
</tr>
<tr>
<td>E0292</td>
<td>urine</td>
<td>USA</td>
<td>1992</td>
<td>200 kb vanA plasmid (rep-Inc18)</td>
<td>HGT (Palmer, Kos and Gilmore 2010)</td>
</tr>
<tr>
<td>H74</td>
<td>hepatic fluid</td>
<td>Portugal</td>
<td>2001</td>
<td>50 kb vanA plasmid (rep-Inc18,rep-pRUM; PSK: ω-ε-ζ)</td>
<td>MDR gene cassette (Rosvoll et al. 2010), inhibit growth (Grady and Hayes 2003), enhance fitness, support plasmid maintenance, horizontal gene transfer (Palmer, Kos and Gilmore 2010)</td>
</tr>
</tbody>
</table>
Figure 1A and 1B. Mean concentrations of *E. faecium* strains held in river water without (A) or with (B) added nutrients. Error bars represent standard error of the mean for five replicates for each strain. Data are provided for strains growing on mEI + vancomycin, with the exception of vancomycin-sensitive 64/3, which was grown on mEI. Culturable *E. faecium* dropped to undetectable levels at Day 7, therefore Day 4 concentrations to calculate the log\(_{10}\) reduction. Note: On Day 2, the mean of quantifiable replicates was used when all five replicates were not quantifiable.
Figure 2. Comparison of log\(_{10}\) reduction at Day 4 for each strain of \textit{E. faecium} in natural conditions (0X) and augmented nutrients (1X). * indicates that a significant difference between the control (64/3) and transconjugant (E0292) was detected (\(P=0.022\)). Error bars represent standard error of the mean.

Figure 3. Correlations of plasmid size and log\(_{10}\) reduction of \textit{E. faecium} in control unamended (A) and nutrient-amended (B) treatments. The correlation is significant for the unamended treatment (\(P=0.0207; r=0.5129\)), but not for the nutrient-amended treatment (\(P=0.3998; r=0.1992\)). Plasmid size for vancomycin-sensitive 64/3 is represented as 0 on the x-axis.
Discussion

The fitness cost of plasmids in general (54) and antibiotic resistance plasmids in particular (33, 55, 56) has been the subject of study for decades. The general conclusion drawn from such studies is that plasmids incur a cost to fitness in laboratory conditions, in the absence of selective pressure. Because growing emphasis is placed on the importance of environmental reservoirs of antibiotic resistance, we designed this study to assess the cost of mobile plasmids carrying the vanA operon to E. faecium fitness in a simulated aquatic environment. We hypothesized that organic nutrient levels could influence the burden of plasmid maintenance, a linkage that has not been well-explored.

In this study, the lack of a direct effect of nutrients on survival may be explained by relatively low nutrient concentration amendments used in the treatments. Nutrient availability is a key abiotic factor that influences bacterial survival in aquatic ecosystems (57-59), specifically organic carbon for Enterococcus spp. (37) and other heterotrophic bacteria. The same organic carbon sources (glucose, pyruvate, acetate) used in this study were also used in previous microcosm studies, where conditions were designed to simulate eutrophic (1X, also used in this study as “augmented” nutrient conditions) and sewage-contaminated systems (5X) (45). Nutrient amendment in the previous work enhanced survival of E. coli, but only at the 5X level (five times the concentration used here). Using more concentrated nutrient treatments in future studies may reveal a relationship or help to disentangle the interaction between strains and nutrient levels, and could better assess the impacts of raw sewage containing VRE released into aquatic ecosystems.
Greater reduction of one plasmid-bearing *E. faecium* strain compared to the parent strain, which lacked plasmids, confirms previous findings of a fitness cost of plasmids (32, 33, 35-37). Two of the transconjugant strains used in this study, E0292 and H182, were used previously to assess the fitness cost in competitive mixed culture experiments (35). The largest plasmid insert had a detrimental effect (E0292; relative fitness = 0.73) and the smallest was beneficial (H182; relative fitness = 1.1). While the microcosms in this study confirm that reduced fitness is associated with the large plasmid, there was no observed benefit to smaller plasmids in surface water microcosms. These results support the hypothesis that large plasmids exert fitness costs to their hosts, although the conditions in the competitive culture experiments contrasted sharply from those in this study, which simulated a natural aquatic environment.

The largest vancomycin resistance plasmid significantly reduced survival in *E. faecium* transconjugant E0292 compared to the vancomycin-susceptible counterpart under natural nutrient conditions in this study. The correlation between bacterial reduction and plasmid size was driven by the presence of any plasmid compared to lack of a plasmid. Log$_{10}$ reduction the other transconjugant VRE strains was not significantly different than that of the parent strain, suggesting that there may be a cut-off or threshold size for larger plasmids to negatively impact survival. While exact copy numbers were not established for these plasmids, their size and previously characterized replicon types indicate that they are large, low-copy number plasmids, with less than 5-10 copies per cell (40, 61). Sequencing, replicon typing and classification systems have revealed a wide variety in the molecular makeup of *Enterococcus* spp. plasmids (62). While the precise genes or gene cassettes responsible for decreased fitness have not been elucidated, the
difference between the large plasmid and the parent strain was only determined in the natural (unaugmented) nutrient conditions, which suggests that the oligotrophic environment affects the relative success of bacteria bearing large plasmids, and that eutrophic nutrient levels may reduce the cost of fitness. The fact that smaller plasmids that confer clinically-relevant VRE characteristics did not exert a cost of fitness in the environmental conditions studies here is cautionary, as it suggests that smaller plasmids may not hamper survival of these pathogens.

Various genes within conjugative plasmids can mediate virulence and survival (64-68), but known genes on the plasmids studied here were not associated with survival advantages or inhibition. Replicon typing of plasmids was previously determined for the VRE trasnconjugant strains used, as shown in Table 1 (40). All three conjugated plasmids are classified as repINC18, repINC-pRUM, or both (40, 69). E0292 and H74 contained a repINC18 plasmid, which is associated with horizontal gene transfer and no known survival advantages (70), while H74 and H182 both contained the repINC-pRUM-type plasmid (see Table 1). Toxin-antitoxin systems of repINC-pRUM plasmids have been shown to inhibit survival and also support plasmid maintenance in *E. coli* (71) but did not appear to affect survival in this study with *E. faecium* when compared to parent strains lacking plasmids. Both transconjugants with the repINC-pRUM plasmids (H74 and H182) had a lower reduction in microcosms compared to the strain not classified as repINC-pRUM (E0292). However, the data presented and analyzed here are insufficient to confirm whether the plasmid types contribute to or inhibit persistence of VRE broadly, or how variability in molecular structure of plasmids influenced survival.
Plasmid maintenance was not affected in any of the VRE strains throughout the experiment, as confirmed by culturing in the presence and absence of vancomycin. These results support previously published data using the same strains where plasmids were maintained in liquid brain heart infusion (BHI) broth in the absence of selective pressure (40). The \(\omega-e-\zeta\) TA system associated with plasmid maintenance was present in one of the plasmids used in this study, H74, but reduction of that strain was not significantly different compared to the parent strain lacking plasmids. Studies with \(E.\ coli\) have shown survival advantages associated with plasmid loss, including increased growth rates in their absence, and that bacteria have a higher propensity to lose larger plasmids (34, 72). However, no similar studies have been performed with plasmids harboring vancomycin resistance in \(E.\ faecium\). Recent studies have contributed to deeper understanding of plasmids and mobile genetic elements within \(Enterococcus\) spp. (69, 71, 73) including assessing the potential for gene transfer in marine sediment (74) and the evolutionary phylogeny of the “mobilome” (75). These studies can contribute to assessing the spread of antibiotic resistance in environmental reservoirs.

This study showed that the clinical \(E.\ faecium\) strain carrying the largest plasmid decayed faster than its plasmid-less counterpart in an aquatic environment with low available nutrients and in the absence of selective pressure. Strains with smaller plasmids persisted as well as the plasmid-less strain. Clinical VRE strains can enter the environment through raw sewage spills and this study investigated the fate of these pathogens in environmental surface water, showing that large plasmids inhibit survival, but that increased levels of organic nutrients can alleviate the cost of plasmid fitness.
References


AFTERWORD

Summary

The research completed and presented in this dissertation provides new knowledge in understanding the ecology of antibiotic resistance. The novel finding of clinically relevant, highly resistant VRE in residential sewage emphasizes the potential danger of sewage spills in spreading antibiotic resistance. The persistence of antibiotic resistance genes associated poultry litter confirms the role of soil and sediment in their maintenance. Mobile genetic elements, including plasmids as discussed in Chapter Four, can be significant factors in survival of clinical strains of antibiotic resistant bacteria in aquatic environments and are important mechanisms that require further study in ecological implications of antibiotic resistance.

Progress and Future Research

Recent research studies use innovative approaches to find practicable solutions to the problem of antibiotic resistance. Antimicrobial peptides, or AMPs, are being developed that can disrupt cell walls of Gram-negative bacteria with minimal or no toxicity to cells of the infected host (1). Studies have also assessed how CRISPR-cas modifications enzyme systems impact the spread of antibiotic resistance genes in enterococci (2). Increased attention and affordability in sequencing are producing new results and perspectives based on on the microbiome and resistome (3-7).
Public health professionals have developed new frameworks to understand and study the ecology of antibiotic resistance, using the OneHealth approach to more holistically address the problem in a broad and applicable context (8-10). Global health policy needs being more clearly defined and emphasized, including setting targets and using current infrastructure more wisely for surveillance (11).

Antibiotic resistance impacts ecosystems and human health and studying the survival of antibiotic resistant bacteria and antibiotic resistance genes outside of hospital settings can contribute to understanding these dynamics. An interdisciplinary and collaborative approach that incorporates monitoring and surveillance associated with clinical relevant targets is essential to developing practical solutions to the problem of antibiotic resistance. Legislative and regulatory initiatives regarding prudent use of antibiotics in human health and animal agriculture are essential to combat the global public health problem.

References


# APPENDIX A

Table 1. Antibiotics relevant to human and animal health, which were assessed in this study

<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>Generic drug names</th>
<th>Uses/target pathogens</th>
<th>Mode of action</th>
<th>Common resistance genes</th>
<th>WHO rating</th>
<th>Refs.</th>
<th>Year of discovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>macrolides</td>
<td>erythromycin, azithromycin, clarithromycin</td>
<td>Staphylococcus spp., Streptococcus spp., <em>Haemophilus influenzae</em></td>
<td>inhibit protein synthesis</td>
<td><em>ermB</em></td>
<td>Critical</td>
<td>(2)</td>
<td>1949</td>
</tr>
<tr>
<td>beta-lactam</td>
<td>penicillin, ampicillin, amoxicillin, methicillin, cephalosporins</td>
<td><em>Mycobacterium tuberculosis</em>, <em>Escherichia coli</em>, <em>Bacillus spp.</em>, <em>Staphylococcus spp.</em>, <em>Streptococcus spp.</em></td>
<td>disrupt cell wall synthesis</td>
<td><em>ampC, mecA, bla[X]</em></td>
<td>Critical</td>
<td>(3, 4)</td>
<td>1929</td>
</tr>
<tr>
<td>glycopeptides</td>
<td>vancomycin, teicoplanin</td>
<td>Gram-positive cocci; <em>Staphylococcus spp.</em>, <em>Enterococcus spp.</em></td>
<td>disrupt cell wall synthesis</td>
<td><em>vanA</em></td>
<td>Critical</td>
<td>(5)</td>
<td>1953</td>
</tr>
<tr>
<td>quinolones</td>
<td>ciprofloxacin, ofloxacin</td>
<td>Broad spectrum; <em>Plasmodium spp.</em></td>
<td>inhibit DNA synthesis</td>
<td><em>qnrS</em></td>
<td>Critical</td>
<td>(6)</td>
<td>1962</td>
</tr>
</tbody>
</table>


APPENDIX B

The attached publication represents Chapter 2 of the dissertation, entitled:
Vancomycin-Resistant Enterococci and Bacterial Community Structure Following a Sewage Spill into an Aquatic Environment.
Vancomycin-Resistant Enterococci and Bacterial Community Structure following a Sewage Spill into an Aquatic Environment

Suzanne Young,a Bina Nayak,a Shan Sun,b Brian D. Badgley,b Jason R. Rohr,a Valerie J. Harwooda

Department of Integrative Biology, University of South Florida, Tampa, Florida, USAa; Department of Crop and Soil Environmental Sciences, Virginia Tech, Blacksburg, Virginia, USAa

ABSTRACT
Sewage spills can release antibiotic-resistant bacteria into surface waters, contributing to environmental reservoirs and potentially impacting human health. Vancomycin-resistant enterococci (VRE) are nosocomial pathogens that have been detected in environmental habitats, including soil, water, and beach sands, as well as wildlife feces. However, VRE harboring vanA genes that confer high-level resistance have infrequently been found outside clinical settings in the United States. This study found culturable Enterococcus faecium harboring the vanA gene in water and sediment for up to 3 days after a sewage spill, and the quantitative PCR (qPCR) signal for vanA persisted for an additional week. Culturable levels of enterococci in water exceeded recreational water guidelines for 2 weeks following the spill, declining about five orders of magnitude in sediments and two orders of magnitude in the water column over 6 weeks. Analysis of bacterial taxa via 16S rRNA gene sequencing showed changes in community structure through time following the sewage spill in sediment and water. The spread of opportunistic pathogens harboring high-level vancomycin resistance genes beyond hospitals and into the broader community and associated habitats is a potential threat to public health, requiring further studies that examine the persistence, occurrence, and survival of VRE in different environmental matrices.

IMPORTANCE
Vancomycin-resistant enterococci (VRE) are harmful bacteria that are resistant to the powerful antibiotic vancomycin, which is used as a last resort against many infections. This study followed the release of VRE in a major sewage spill and their persistence over time. Such events can act as a means of spreading vancomycin-resistant bacteria in the environment, which can eventually impact human health.

Antibiotic-resistant bacteria (ARB) are a growing public health threat and an economic burden globally. The Centers for Disease Control and Prevention (CDC) in the United States has placed a high priority on addressing antibiotic resistance because of rising rates of ARB infection and associated disease burden and health care costs (1, 2). Most infections caused by ARB are nosocomial transmissions (i.e., originating in a hospital), but the role of environmental reservoirs in spreading ARB outside clinical settings is poorly understood. Studies have emphasized the role of environmental reservoirs in the spread of antibiotic resistance for decades, but more field and laboratory studies are necessary to address the specific mechanisms and conditions under which ARB survive and antibiotic resistance genes (ARGs) persist or can be transferred (3–5). Wastewater treatment plants (WWTPs) are sources of ARB, ARGs, and antimicrobial compounds through both treated effluent and the unplanned release of raw sewage to surface waters (6–9). ARB, ARGs, and antibiotics can be released into aquatic environments through human and agricultural waste, establishing routes of human exposure and threats to ecosystem health.

Vancomycin is a glycopeptide antibiotic that is used to treat infections caused by Gram-positive bacteria. It is considered a drug of last resort because of its historical success with the most recalcitrant infections caused by Gram-positive bacteria (10, 11). When vancomycin is rendered ineffective (i.e., when target bacteria are resistant), therapeutic treatment may fail and infections can be fatal (12, 13). Intrinsic, low-level resistance to vancomycin is characteristic of Enterococcus casseliflavus and Enterococcus galinarum but is of less clinical concern than acquired, high-level vancomycin resistance (≥32 µg · ml⁻¹) (14). Acquired vancomycin resistance can occur through the transfer of mobilizable genetic elements (15–17). Nine genes that confer vancomycin resistance in enterococci have been described, eight of which can be acquired (18). The most concerning from a public health perspective is the vanA gene, which is linked to most infections with human vancomycin-resistant enterococci (VRE). vanA is usually carried on a plasmid-borne transposon (Tn1546) (19–21) and confers high-level resistance to vancomycin (>64 µg · ml⁻¹) (22).

The use of the glycopeptide avoparcin in animal agriculture in Europe has been linked to clinical vancomycin resistance (23–25). Although glycopeptides have not been used in animals in the United States, clinical incidence of VRE has steadily increased in past decades (2, 18, 26–29). Detection of VRE in the United States...
Young et al.

has been predominantly in clinical cases and hospital sewage (30, 31). The monitoring of VRE and associated resistance genes outside the hospital setting is necessary to better understand the spread of resistance and the increased risk to public health (6). Previous studies in Europe and Australia have reported community spread of VRE and fecal colonization of nonhospitalized individuals, but this has not been shown in the United States (32–35).

Antibiotic resistance can spread in bacterial habitats in the external environment, where antibiotics, ARB, and ARG enter water and sediments (6). The influx of sewage-associated microbes and other allochthonous bacteria into an aquatic environment can have ecological impacts, affecting community structure, nutrient cycling, and other ecosystem processes (36–38). In addition, the dynamics of gene exchange in microbial communities can be altered, and transfer of resistance genes may occur (39, 40). VRE and vancomycin resistance genes have been detected globally in the feces of agricultural and wild animals (30, 41–44), surface waters (45–47), WWTPs (48), domestic (community) sewage (49), and hospital sewage (30, 46, 50). Clinically relevant strains and vanA genes have rarely been reported in the environment in the United States (51, 52). The prevalence of genes encoding vancomycin resistance in the environment may increase the frequency of transfer to other Gram-positive pathogens (53), including the opportunistic pathogen Staphylococcus aureus (54). The incidence of vancomycin-resistant S. aureus (VRSA) in hospitals is low; however, 13 incidences have been reported in the United States as of 2014 (55), and the emerging threat is a concern for public health.

Relatively little information is available about the prevalence of clinically relevant VRE and vanA genes in aquatic environments, but many studies that have attempted to detect them have failed to find them in relatively pristine environments. Studies around the world have infrequently and inconsistently detected vanA genes and Enterococcus species isolates with vanA phenotypes in WWTP effluent and surface waters (56–59). One study in the United States isolated Enterococcus faecium carrying vanA genes on a recreational marine beach in Washington (52), but no other confirmation has been established outside hospital settings. In this field study, culturable VRE and/or vanA genes were detected in sediment and water samples after a sewage spill released more than 500,000 gallons of untreated sewage in a residential neighborhood. Illumina next-generation sequencing (NGS) of environmental DNA from sediment and water revealed the temporal changes in the microbial community after a major influx of untreated sewage.

MATERIALS AND METHODS

Sample collection. A sewer line break in Pinellas County, FL, released more than 500,000 gallons of untreated sewage into a neighborhood drainage ditch beginning 27 September 2014. The line break was repaired with a bypass valve on 30 September 2014 after the sewage leakage was diverted. The site was also washed down, vacuumed, and disinfected with lime. A well-point system was also installed at the site to dewater, which resulted in groundwater discharge. Well-point systems are commonly used in engineering and construction and consist of a series of vacuum pumps designed to draw water up out of the ground. The ditch is connected to estuarine waters through wetlands. Photos of the site are included in Fig. S3A and B in the supplemental material. Water and sediment samples were collected at the spill site, along the drainage ditch for a distance of 800 m, and in adjacent receiving waters. Samples were collected seven times over the course of 7 weeks after the spill (1 October 2014 to 21 November 2014), to determine the persistence of sewage-associated microbes and VRE in the environment.

Six sites (NC-01, NC-02, NC-03, NC-04, NC-05, and NC-06) were selected for spatial assessment, but the majority of reported results are limited to one site that was sampled on all dates, NC-03. The additional sites where early sampling occurred are noted in the maps provided in Fig. S1 in the supplemental material. Site NC-01 became inaccessible after the first 2 weeks of sampling because it was filled in by construction crews. We were not able to collect sediment at the boat ramp in any instance because the site was a dock surrounded by mangroves. The boat ramp was included to represent recreational waters that may have been impacted by the spill. Water samples were collected in 500-ml sterile containers. Sediment samples were collected using a 50-ml sterile, screw-cap tube to scoop up the top 1 to 2 cm of sediments. All samples were transported on ice to the laboratory and processed within 6 h. Enterococci were also quantified by the Pinellas County Water and Sewer Department staff at 16 sites (see Fig. S2A and B in the supplemental material) near the point of the line break for 12 days using standard methods (ASTM D6503-99).

Isolation of and confirmation of VRE. Water and sediment samples were processed using membrane filtration according to U.S. Environmental Protection Agency (EPA) Method 1600 for culturable enterococci (60), with modifications for the detection of VRE. Water samples were processed in multiple volumes (1 to 300 ml) on each sampling date over the course of the sampling period to account for variability in enterococcal concentrations. Vancomycin stock solution was prepared as an aqueous solution from sodium salt (Acros Organics/Thermo Fisher Scientific, NJ, USA) and sterile nuclease-free water to a final concentration of 10 mg · ml−1 and filter sterilized. To detect culturable VRE, Enterococcus indoxyl-β-d-glucoside (mEI) agar (Becton Dickinson, Sparks, MD) was prepared according to the manufacturer’s recommendations. After the medium cooled to 55°C, the vancomycin solution was added to a final concentration of 32 µg · ml−1, the breakpoint for full resistance (14, 61). Sediment samples (30 g wet weight) were diluted 1:10 in phosphate-buffered saline (PBS) and hand shaken for 2 min to detach bacteria from particles (62). Sediment samples of the diluted buffered solution were processed in volumes from 0.1 to 100 ml depending on the sampling date and on previous concentrations of enterococci. Multiple dilutions for water and sediment were processed on each date to obtain viable colony counts.

To confirm culturable VRE as enterococci harboring the vanA gene, colonies with blue halos that grew on vancomycin-amended mEI were transferred to enterococcosel broth (EB) using sterile pipet tips or sterile toothpicks and were grown for 24 h. Wells that turned black were streaked onto tryptic soy agar (TSA) (Becton Dickinson, Sparks, MD) and then isolated again onto vancomycin-amended mEI (32 µg · ml−1). Isolated colonies were grown overnight in 5 ml Luria-Bertani (LB) broth (Thermo Fisher Scientific, Waltham, MA) that was amended with 32 µg · ml−1 vancomycin. DNA was extracted from overnight cultures using a GenElute bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO). Nucleic acid concentration was measured using a NanoDrop spectrophotometer to confirm successful extraction, and DNA was stored at −20°C in aliquots. Quantitative PCR (qPCR) was carried out with an Applied Biosystems 7500 real-time PCR system to confirm isolates as Enterococcus spp. (63) carrying the vanA gene (64). Isolates were identified to the species level by DNA sequencing of the 16S rRNA gene using universal bacterial primers (8F, 1492R) to amplify the 16S rRNA (65, 66); the PCR product was then purified using a GeneJet PCR purification kit (Thermo Fisher Scientific, Waltham, MA), sequenced by Eurofins Genomics (Huntsville, AL), and identified to the genus and species levels by using BLAST to reference the GenBank database (NCBI).

Sequencing and molecular analysis of environmental DNA. Water (500 ml) was also filtered to obtain environmental DNA, and filters were stored at −80°C for DNA extraction. Sediment samples were also stored for DNA extraction. DNA from environmental water and samples was extracted and purified using the Mo Bio PowerWater kit from 0.45-µm
filters. DNA from environmental sediment samples was extracted using Mo Bio PowerSoil kits directly from 0.3-g samples of sediment (Mo Bio Laboratories, Carlsbad, CA). Bacterial communities in those samples were characterized by sequencing the V4 region of the 16S rRNA gene. PCR was carried out to amplify the V4 region with the 515F and 806R primer pair, which included sequencer adapter sequences for Illumina sequencing (67, 68). The forward primer also contained a 12-bp barcode sequence unique to each sample. Each 25-µl PCR mixture contained 12 µl of PCR Water (Mo Bio Laboratories, Carlsbad, CA, USA), 10 µl of 2.5× 5 Prime HotMasterMix (Gaithersburg, MD), 1 µl of each of the primers (5 µM), and 1 µl of template DNA. The conditions for PCR were as follows: 94°C for 3 min, 35 cycles at 94°C for 45 s, 50°C for 60 s, and 72°C for 90 s, and a final extension of 10 min at 72°C. Amplicons were amplified using PicoGreen (Invitrogen) and a plate reader (Infinite 200 Pro; Tecan) and were then pooled in equimolar ratios. This pool was cleaned using the UltraClean PCR clean-up kit (Mo Bio) and sequenced in an Illumina MiSeq run (2 × 150 bp) at Argonne National Laboratory. Sequencing reads were processed using QIIME (69) and USEARCH (70). The forward and reverse reads were merged, and then the merged reads were demultiplexed and filtered with a minimum Phred quality score of 20. Filtering resulted in about 388,000 high-quality reads, averaging about 28,000 reads per sample. Those reads were then clustered into 1,685 operational taxonomical units (OTUs) with a 97% similarity threshold. Chimeric sequences were identified with UCHIME and removed from OTUs (71). The taxonomy of the OTUs was assigned an RDP classifier against the SILVA databases (72, 73). For all downstream analyses, 10,000 reads were randomly selected per sample to correct for differences in sequencing depth.

Quantitative PCR (qPCR) was carried out with an Applied Biosystems 7500 real-time PCR system based on a previously published protocol for the vanA gene (64). Targets in environmental DNA were amplified using the following master mix composition per 25-µl reaction mixture: 12.5 µl TaqMan environmental master mix 2.0 (Thermo Fisher Scientific, Waltham, MA), 3 µl primer/probe mix (composed of 74.5 µM of each primer at 100 µM and 6 µl of target probe at 100 µM), 2.5 µl bovine serum albumin (BSA) (2 mg · ml⁻¹), 2 µl sterile nuclease-free water, and 5 µl template DNA. Temperature cycling consisted of 2 min at 50°C, 10 min at 95°C, and then 40 cycles of 15 s at 95°C and 1 min at 60°C. The lower limit of quantification (LLOQ) for the qPCR assay was 2.5 gene copies per reaction based on successful amplification in 50% of replicates of the lowest concentration on the standard curve (74). Sample LLOQ was 1.67 × 10⁴ gene copies per 100 g for sediment samples and 10 gene copies per 100 ml for water samples. Blanks containing sterile nanopure water in place of a sample were processed as negative controls (no-template controls [NTCs]). No blank amplified in any vanA PCR assay. When the quantification cycle (Cq) values for the two replicates were greater than the Cq values for the LLOQ, results were reported as not detected (ND). Samples where neither replicate amplified and samples that did not successfully amplify in the two replicate qPCRs (amplified in 1 of 2) were reported as not detected (ND). The standard curve for vanA was constructed using a synthetic plasmid (IDT, Coralville, IA), containing the target sequence of the pIP816 vanA plasmid as previously published (NCBI accession number X56895) (64). Inhibition of amplification in environmental samples was tested using a qPCR SYBR green assay for the vvhA gene of Vibrio vulnificus (75). V. vulnificus is an autotrophic marine bacterium that does not grow in freshwater environments. Reaction mixtures contained 4 µl of DNA sample and 1 µl of V. vulnificus DNA (20,000 copies) and were compared to a control reaction mixture containing 4 µl of nuclease-free water and 1 µl of V. vulnificus DNA (20,000 copies) of previously published cycling conditions and primers (76).

Accession number(s). Sequences were deposited in the NCBI BioProject database under BioProject accession number PRJNA322710.

RESULTS

Concentrations of culturable enterococci in water were high at the site of the spill (NC-03) immediately after the event (4.2 × 10⁵ CFU per 100 ml), exceeding the U.S. EPA sample threshold value (STV) standards for recreational waters of 1.3 × 10² CFU per 100 ml (77) (Fig. 1). Levels decreased over time but did not fall below 1.3 × 10² CFU per 100 ml at NC-03 until 30 October 2014, more than 1 month after the event (Fig. 1). Enterococcal levels at the boat ramp in receiving marine waters approximately 3 km from the spill were within regulatory limits at each sampling date, ranging from 5 to 22 CFU per 100 ml. Enterococcal levels were 2 to 3 orders of magnitude higher in sediment than in water at NC-03 and also decreased over time (Fig. 1).

Enterococci were also monitored by Pinellas County at eight surface water sites ranging from 1 to 9 km away from the spill (B, C, F, G, H, I, J, and Q) for 12 consecutive days following the spill (see Fig. S2A and B in the supplemental material). Four sites within 4.5 km (from near to far, B, C, J, and Q) displayed enterococcal levels that exceeded recreational water quality standards (130 CFU per 100 ml) (77) for some duration after the spill. Exceedances were recorded at site B for 8 days, site C for 1 day, site J for 2 days, and site Q for 1 day. Maximum enterococcal levels were recorded 6 days after the spill at site B (2,100 CFU per 100 ml) and 1 day after the spill at site C (210 CFU per 100 ml), site J (160 CFU per 100 ml), and site Q (170 CFU per 100 ml). These sites were in the receiving waters directly adjacent to the site of the spill, Long Bayou and Cross Bayou, with the exception of site Q, which was in Boca Ciega Bay. Recreational water quality standards were not exceeded at the other four sites where enterococci were measured (F, G, H, and I) in Boca Ciega Bay, a body of water that mixes with the Gulf of Mexico and that is more than 5 km away from the spill.

VRE were detected by culture and confirmed as Enterococcus faecium in water collected 2 and 3 days after the spill ceased at NC-01, NC-02, and NC-03 (1 October 2014 and 2 October 2014) but could not be confirmed in water or sediment on subsequent dates (Fig. 1). A subset of putative VRE isolates from water sampled on 1 October 2014 and 2 October 2014 (11 of 15) was iden-
tified as *E. faecium* by 16S rRNA gene sequencing. The qPCR assay for *vanA* also confirmed that all 11 isolates identified as *E. faecium* carried the *vanA* gene. The other four putative VRE isolates were identified as *Pediococcus* spp. by 16S rRNA sequencing. Colonies that grew on mEI amended with 32 µg · ml⁻¹ vancomycin but could not be isolated and confirmed with molecular analyses were detected in water in October 2014 and in sediment until 16 October 2014.

The *vanA* gene was detected in environmental DNA samples extracted from water and sediment up to 12 days after the spill (9 October 2014) at the sites within 800 m of the spill (NC-01, NC-02, and NC-03) (Fig. 1; Table 1) but not at later dates. Concentrations of *vanA* gene copies were approximately two orders of magnitude higher in sediment than in water (Table 1) but were reported per 100 g (wet weight) versus per 100 ml. In water, the maximum for *vanA* gene copies was 2.2 log₁₀ gene copies per 100 ml (at site NC-01 on 9 October 2014), and the average was 1.9 log₁₀ gene copies per 100 ml. In sediment, the maximum for *vanA* gene copies was 5.0 log₁₀ gene copies per 100 g (at site NC-03 on 2 October 2014), and the average was 3.9 log₁₀ gene copies per 100 g.

Sequencing results from environmental DNA on seven sampling dates where both sediment and water were collected showed distinct bacterial communities in water and sediment samples. In both matrices, dates closest to the spill (2 October 2014 and 9 October 2014 for sediment and water plus 16 October 2014 for water) were distinctly separate from those in the later sampling weeks (Fig. 2). The trend shown by these data suggests that the sediment and water at this site took approximately 2 to 3 weeks to return to a stable structure following the spill. The change in community composition is supported by a similar time frame of noticeable sewage impacts on fecal indicator bacteria and VRE (Fig. 1).

Six bacterial families shown to be highly prevalent in domestic sewage in the United States (*Bacteroidaceae*, *Ruminococcaceae*, *Lachnospiraceae*, *Porphyromonadaceae*, *Veillonellaceae*, and *Prevotellaceae*) (78) decreased in frequency with respect to total 16S rRNA sequences over time at NC-03 in sediment and water (Fig. 3). OTUs identified to the family level represented 85% to 95% of the total OTUs, with the exception of sediment on 2 October 2014, where 43% were identified to the family level. One of the sewage-associated families, *Porphyromonadaceae*, was found in the dominant taxa (top 10 most abundant) on the first sampling date and not at any later dates. Alpha diversity did not reveal temporal trends during the course of the sampling. The temporal trend of sewage-associated families also aligns with trends demonstrated in enterococci, VRE, and community structure (Fig. 1 to 3). Dominant families in sediment were different from dominant families associated with the gut flora of mammals, and *Comamonadaceae*, a family containing common environmental denitrifiers, had the greatest decline in relative abundance in water from the first to later sampling dates. Similar trends in the distinction between microbial communities in water and sediment were observed in taxonomic diversity based on phyla (see Fig. S4A and B in the supplemental material). Families containing common pathogens associated with the gut flora of mammals, and *Comamonadaceae*, a family containing common environmental denitrifiers, had the greatest decline in relative abundance in water from the first to later sampling dates. Similar trends in the distinction between microbial communities in water and sediment were observed in taxonomic diversity based on phyla (see Fig. S4A and B in the supplemental material).

### Table 1 Detection and levels of *vanA* measured by qPCR in water and sediment at three sites near the origin (within 800 m) of the sewage spill over eight sampling dates

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Site</th>
<th>Days postspill</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Water (log₁₀ gene copies per 100 ml)</td>
<td>NC-01</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>NC-02</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>NC-03</td>
<td>1.92</td>
</tr>
<tr>
<td>Sediment (log₁₀ gene copies per 100 g)</td>
<td>NC-01</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>NC-02</td>
<td>4.24</td>
</tr>
<tr>
<td></td>
<td>NC-03</td>
<td>4.95</td>
</tr>
</tbody>
</table>

*Sample limits of detection were 4 gene copies per 100 ml water and 6.7 × 10² gene copies per 100 g sediment. Day 1 postspill is considered to be 1 October 2014. Note that access issues prevented sampling at all sites on all dates; data analysis focuses on site NC-03 where samples were collected on each sampling date.*  

**FIG 2** Analysis of DNA sequencing of the 16S rRNA gene in water and sediment on seven dates at site NC-03. The blue gradient represents water samples, and the red gradient represents sediment samples; the color gradient represents different time points in either water (W) or sediment (S) where darker shades are immediately after the spill or earlier in time.
(Enterobacteriaceae and Enterococcaceae) were present at low levels in water and sediment throughout the study (see Fig. S5A and B in the supplemental material) and were combined to represent an average of 0.41% and 1.4% of sequence reads over time in water and sediment, respectively.

**DISCUSSION**

The sewage spill that we studied corresponded with elevated levels of enterococci, VRE, and vanA genes in water and sediment, indicating their release into the environment. All of these levels diminished steadily over the 2 weeks following the spill. No vanA genes were detected in environmental samples after 12 days at the site of the spill. This observation, and the fact that high-level VRE have been infrequently observed in uncontaminated surface waters (30), indicates that their presence in the environment before the spill is unlikely and that these contaminants were sewage associated (i.e., no background levels of vanA or VRE would be expected in the environment). The mitigation measures taken after the spill (vacuum pumping, washing out, lime treatment) probably decreased levels of microorganisms from sewage but left high levels of enterococci that slowly diminished over time in the area directly adjacent to the spill. The plume of the sewage spill was also indicated by the broader sampling effort in the region (as processed by Pinellas County), where enterococcal levels exceeded recreational water quality standards at the site closest to the spill (site B) but decreased after 8 days. Sites downstream from the spill where enterococcal levels were high decreased after 1 to 2 days. Flow rates, temperature, and other environmental conditions may impact the persistence and reach of contamination, but these factors were beyond the scope of this study.

The transfer of resistance through mechanisms such as horizontal gene transfer, demonstrated by the detection of the mobile vanA gene, can impact human health and the spread of resistance in the environment. This study has demonstrated the release of potentially pathogenic VRE and vanA genes into surface waters by sanitary sewer overflow in the United States. High-level VRE and vanA genes have been found in sewage from a hospital in Florida but were not found in other sewage samples that were not directly associated with a hospital (30). The spill in this study was not in close proximity to any hospital; the closest is 2.6 miles from the site of the sewer line break, and sewage from the hospital flows away from the break site. Previous studies have also investigated VRE in aquatic ecosystems, sanitary sewage, and WWTPs (48, 79–81), but community sewage (not associated with a hospital) has not been explicitly linked to vanA genes or highly resistant VRE in the United States. Results confirmed that untreated residential sewage released into aquatic environments can potentially be a route of human exposure to ARB and contribute to environmental reservoirs of ARB and ARGs.

Colonies that resembled VRE were detected in water samples through 30 October 2014 and in sediment samples through 16 October 2014; however, putative VRE colonies observed after 2 October 2014 could not be isolated based on the methods described above for confirmation. In all probability, they were either Enterococcus species or members of other genera that could “struggle” at 32 μg·ml⁻¹ vancomycin on a crowded plate but did not possess vanA and so could not grow when subcultured on vancomycin. This observation reemphasizes the inaccuracy implicit in reporting VRE solely based on culture methods as further evidenced by the identification of Pediococcus spp. in this study. Other studies have demonstrated the isolation of a small percentage of genera other than Enterococcus on mEI (47, 82). The addition of vancomycin in the screening step tends to exacerbate the issue, as selection for intrinsically resistant genera, such as Pediococcus, Weissella, and Leuconostoc, also occurs (30).

DNA sequencing analysis has explored the dominant microbial taxa associated with sewage and human feces (78, 83), but the microbial community in waters impacted by sewage has received
less attention. The advantages of this site included limited water input following the initial flushing so that changes in the community could be followed over time without the dilution effect that would occur in a large water body. The influx of sewage at this site produced a bacterial community with a prominent component of sewage- and fecal-associated bacteria that was detectable at the site for at least 2 weeks. The abundance of sewage-associated families declined on a similar time scale to enterococci, but the fate (i.e., death, transport, consumption by predators) of these bacteria and other pathogens was not determined. Some families containing pathogenic members and fecal indicator bacteria (FIB) (Enterococcaceae, Enterobacteriaceae) were represented throughout the sampling period.

Differences in community structure in sediment versus water were evident. The dominant phyla in water were consistent with those found in a study of 10 sites in the Mississippi River (Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, and Verrucomicrobia accounting for approximately 94% of sequences) (84). However, Firmicutes (containing pathogen taxa) were more prevalent in water on the days immediately after the sewage spill than at later dates compared to the consistent low levels in the Mississippi River samples. The dominant taxa in sediment were consistent with published research where Proteobacteria and Firmicutes are prevalent phyla (85). It is interesting to note that the communities in water and sediment changed over approximately the same time frame and that they also remained distinct from one another. The relative rate of change in various environmental habitats bears further exploration, particularly given the extensive literature discussion about the potential role of sediments as environmental reservoirs for microbial pathogens and indicators (86–89).

This study confirms that potentially pathogenic ARB and associated ARGs can be released into the environment through untreated sewage and can persist for days or weeks after the initial introduction. Although the study area was flushed with water immediately after the spill, the sewage signal, as measured by enterococci, declined on a similar time scale to enterococci, but the fate (i.e., death, transport, consumption by predators) of these bacteria and other pathogens was not determined. Some families containing pathogenic members and fecal indicator bacteria (FIB) (Enterococcaceae, Enterobacteriaceae) were represented throughout the sampling period.

Differences in community structure in sediment versus water were evident. The dominant phyla in water were consistent with those found in a study of 10 sites in the Mississippi River (Proteobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, and Verrucomicrobia accounting for approximately 94% of sequences) (84). However, Firmicutes (containing pathogen taxa) were more prevalent in water on the days immediately after the sewage spill than at later dates compared to the consistent low levels in the Mississippi River samples. The dominant taxa in sediment were consistent with published research where Proteobacteria and Firmicutes are prevalent phyla (85). It is interesting to note that the communities in water and sediment changed over approximately the same time frame and that they also remained distinct from one another. The relative rate of change in various environmental habitats bears further exploration, particularly given the extensive literature discussion about the potential role of sediments as environmental reservoirs for microbial pathogens and indicators (86–89).

ACKNOWLEDGMENTS

We thank Michelle Maccini of the Pinellas County Utilities Department for sharing data collected at additional sites, as well as the maps indicating their locations. We also thank undergraduate students Rochelle Etienne and Jenna Hindsley for their assistance processing samples and members of the Rohr Lab and Harwood Lab who provided useful comments on the manuscript. Anthony Carbo of St. Petersburg, FL, created the map of the sewage spill area, which is included as supplementary information. Sarah Owens of Argonne National Laboratory provided assistance with processing and method description for the Illumina sequencing.

REFERENCES


community in The Netherlands. J Clin Microbiol
Buiting AG, van Duin A, Verbrugh HA.
enterococci in Australia. Emerg Infect Dis
Healthcare Safety Network at the Centers for Disease Control and Preven-
Nationwide Healthcare Safety Network Facilities.
resistant enterococci from North America and Europe: a report from the
Antimicrobial resistance and molecular epidemiology of vancomycin-
m innocence in the swine sector. J Antimicrob Chemother
m resistance in E. coli. Antimicrob Resist Infect Control
m resistance in Enterococcus faecalis StE6-vanB2 and E. faecium ST95-vanA in faecal samples of wild
1016/j.vetmic.2015.02.025.
1111/1462-2920.12213.
2842.2002.
Roberts MC, No DB, Marzullf JM, Delap JH, Turner R. 3 February 2016. Vancomycin resistant Enterococcus spp, from cows and their environ-
2672.2009.04207.x.
Figure S1. Map of study area
Figure S2A: Map of study area showing additional sampling sites.
Figure S2B. Map of study area: Additional sampling sites
Figures S3A and B. Photos looking east (A) and west (B) at the study site, on the south side of 62nd Avenue North, taken on October 1, 2014.
Figure S4A and B. Taxonomic diversity data based on phyla in water and sediment samples over time. Dominant phyla shown in green and orange are Proteobacteria and Firmicutes, respectively.
Figure S5A and B. Relative abundance of families associated with enteric pathogens (Enterococcaceae and Enterobacteriaceae) in both sediment and water over time. Note that in Panel A, values were zero for Enterococcaceae in water on 10/2/2014, 10/30/2014, 11/13/2014 and 11/21/2014, and are not shown.