METAGENOMIC INVESTIGATION OF ANTIBIOTIC RESISTANCE IN COASTAL MARINE ECOSYSTEMS

A Thesis

by

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BS, Texas A&M University-Corpus Christi, 2014

MASTER of SCIENCE

in

MARINE BIOLOGY

Texas A&M University-Corpus Christi
Corpus Christi, Texas

August 2017

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This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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ABSTRACT

Bays adjacent to developed land are sinks for runoff and municipal wastewater. These inflows carry residual antibiotic compounds and antibiotic-resistant bacteria (ARB) that pose a threat to human and environmental health. This study aims to investigate the abundance and richness of antibiotic resistance genes (ARGs) in Copano Bay, Galveston Bay, Nueces Bay, and 1852 Pass, Texas. Each water body receives a different type of major wastewater inflow dependent on the surrounding land use, and each inflow likely carries a unique suspension of chemical inducers of ARG development. This study proposes that oysters, which can bioaccumulate contaminants from the surrounding water, are an indicator species for the investigation of antibiotic resistance in the coastal environment. Bacterial metagenomes of oyster stomach contents and overlying water were collected and sequenced using Illumina HiSeq technology. The raw sequence reads were filtered for quality and length. The 16S rRNA reads were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) and the Metagenome Analyzer (MEGAN) to determine the taxonomic composition of the microbial communities. The filtered reads were also compared against the Microbial Ecology Group Antibiotic Resistance (MEGARes) database to determine the abundance and richness of ARGs. The dominant bacterial classes in each sample reflected the major wastewater inflow and the surrounding land use. Further, the oyster bacterial communities differed between (but not within) sites while the free-living bacterial communities were very similar across all sampling sites. These findings suggest that local conditions select for distinct oyster microbiota, but those same conditions do not select for distinct free-living microbiota. The abundance of ARGs ranged from 28 to 826 genes while the richness of ARGs ranged from 10 to 32 distinct types. The most abundant ARGs were the

mutated *tuf*, *rpoB*, and *gyrB* genes. Overall, fewer ARGs were detected in the oyster samples compared to the water samples, indicating that oysters were buffered from the bioaccumulation of ARGs. Data describing the prevalence of ARGs – a promising proxy for coastal sewage pollution – can aid officials in managing healthy coastal ecosystems and safeguarding human health.

ACKNOWLEDGEMENTS

I thank my thesis advisory committee chair, Dr. Jeffrey W. Turner, and my thesis advisory committee members, Dr. Gregory W. Buck and Dr. Jennifer Beseres Pollack, for their guidance and expertise throughout the course of this research project. Thanks also go to Dr. John Schwarz and Mona Hochman (Texas A&M University-Galveston Seafood Safety Laboratory), Robert F. Duke (Texas A&M University-Corpus Christi Center for Coastal Studies), Kelley Savage, Danielle Zimmermann, Natasha Breaux, and Terry Palmer (Texas A&M University–Corpus Christi Coastal Conservation and Restoration Ecology Laboratory), and Lee J. Pinnell (Texas A&M University-Corpus Christi Laboratory for Environmental and Microbial Genomics) for their assistance with sample collection and processing. I am especially grateful to my friends and family for their continued support during my time as a graduate student, namely my partner Katelyn Kosted and my wonderful mother Gloria Wendy Dodson.

This research project would not have been possible without funding from Texas A&M University–Corpus Christi (in the form of new laboratory start-up funds) and the Texas Sea Grant Grants-In-Aid of Graduate Research Program.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES PAGE

LIST OF TABLES

TABLES PAGE

CHAPTER I: INTRODUCTION

The rise of antibiotic-resistant bacteria (ARB) is an emerging crisis that poses a significant threat to global human health (1, 2). With the introduction of each new antibiotic drug, resistant bacteria emerge and spread soon after (3). In the United States (U.S.), it is estimated that over 63,000 patients die from nosocomial infections each year (4). Additionally, the economic costs associated with treating ARB infections are high, with estimates approaching $20 billion in healthcare expenses and an additional $35 billion in productivity losses (5). In particular, hospitals have reported a significant increase in nosocomial infections caused by ARB such as vancomycin-resistant *Enterococcus faecalis*, methicillin-resistant *Staphylococcus aureus*, and multidrug-resistant (MDR) *Acinetobacter baumannii* (6). These infections have been increasingly difficult to treat and some recent infections have been essentially untreatable.

Numerous studies have investigated the prevalence of ARB in hospitals and other built environments (e.g., nursing homes, daycare facilities, and wastewater treatment plants), but less is known about the prevalence of ARB in natural environments (7-11). Although ARB occur naturally, they can also enter aquatic environments via point and non-point sources of sewage pollution, which include wastewater treatment plant (WWTP) outfalls, sanitary sewer system leaks and overflows, discharges from boats and marinas, as well as urban and agricultural runoff (2, 9, 12-16). As a result, aquatic environments have been characterized as sinks for sewage-associated ARB and recent studies have detected high levels of antibiotic resistance genes (ARGs) in rivers, lakes, and bays (13, 16-21).

Point and non-point sources of sewage pollution can also load aquatic environments with residual antibiotics as well as other pharmaceuticals, heavy metals, halogenated pollutants, and toxic compounds (16, 22, 23). The addition of these compounds is important in that their

presence can select for the survival and persistence of ARB (16). For example, residual antibiotics can select for the growth of resistant bacteria that may persist in the environment long after these residual pharmaceuticals have dissipated (24). Similarly, heavy metals have been found to strongly correlate with the abundance of the class 1 integrase gene, a mobile genetic element involved in the transfer and maintenance of ARGs in the environment (16, 25).

This combined loading of ARB and compounds that select for their growth is thought to be responsible for the recent emergence of resistance in aquatic fish pathogens such as *Aeromonas salmonicida*, *Vibrio salmonicida*, and *Citrobacter freundii* (26). These pathogens and many others, such as *V. alginolyticus* and *V. harveyi*, cause extensive mortality in aquacultured and wild fisheries (27). Indeed, bacteria cause approximately 34% of all infectious diseases of wild marine fish (27). Invertebrate fisheries have also been impacted by ARB. Shrimp pathogens, such as *V. splendidus* and *V. harveyi*, have increasingly been found to harbor resistance to multiple antibiotic compounds, a phenomenon thought to be exacerbated by the widespread sub-therapeutic use of antibiotics in aquaculture (28).

The combined loading of ARB and ARB-selective compounds is also a threat to human health (through seafood consumption and contact recreation), especially in aquatic environments that are proximal to large, growing population centers (29, 30)(12, 13). In the U.S., vibriosis, which is usually contracted from seafood and coastal water recreational activities, alone causes an estimated 80,000 illnesses each year (31). Coastal population growth is thought to increase coastal anthropogenic pollution, which is correlated with the prevalence of ARB in the coastal environment (32). Consequently, people who use anthropogenic pollution-impacted coastal environments for recreational activities and as food sources are at a heightened risk of contracting ARB infections (33). According to the 2013 U.S. Census Report, 39% of the

population (roughly 123 million people) is concentrated in coastal counties, which comprise less than 10% of the U.S. land area (34). Similarly, people living along the Texas coast account for approximately 25% of the state's population, and coastal populations are growing at 1.9%, making the Texas coast the third most rapidly growing coastline in the U.S. (35).

The future threat of ARB is also likely to increase in response to climate change, namely warming surface water temperatures, as most clinically relevant bacterial are mesophilic (36). According to the National Oceanic and Atmospheric Administration (NOAA), between December 2016 and February 2017, the Texas coast experienced record-high temperatures for the three-month time period. These numbers were the highest recorded in 122 years of data collection. These record high temperatures are exacerbated by the hydrologic conditions of Texas' coastal environments, which are generally shallow and semi-closed with long residence times, creating incubator-like conditions for the growth of mesophilic bacteria (37-40). Increasing temperatures expand seasonal windows for certain infections, such as vibriosis, which is most often contracted during the summer months (41). Additionally, winter temperatures are on a long-term rise (42).

Historically, the detection of ARB was a straightforward two-step process that involved culturing a bacterium and testing it for antibiotic susceptibility (e.g., the Kirby-Bauer Disk Diffusion Susceptibility Test and Minimum Inhibitory Concentration assays) (43). However, the detection of ARB in the natural environment is complicated by an overabundance of bacteria (≈ 99.6%) that cannot be grown in culture (44, 45). Thus, investigations of ARB in natural environments can benefit from a culture-independent approach such as metagenomics. The term "metagenomics" was coined by Dr. Jo Handelsman in reference to the analysis of microbial assemblages made possible through en masse nucleic acid sequencing (46). More recently, the

science of metagenomics has emerged as a powerful tool for investigating antibiotic resistance in natural environments (19, 46-48).

In this study, we sequenced the bacterial metagenomes from the stomach contents of Eastern oysters (*Crassostrea virginica*) and the ambient water overlying said oyster reefs. Suspension-feeding biota, especially bivalve mollusks (e.g., clams, oysters, mussels, and scallops), concentrate both natural and sewage-associated bacteria in their mantle tissues, gastrointestinal tracts, and gills (49). Consequently, oysters can be reservoirs and vectors for pathogenic bacteria and viruses (50, 51). Between 1973 to 2006, food-borne illnesses contracted from mollusks accounted for 45.2% of seafood-related infections (52). Pathogens commonly transmitted via oysters include bacteria (e.g., *Vibrio* spp., *Salmonella* spp., *Staphylococcus* spp., and *Shigella* spp.), viruses (e.g., hepatitis A and norovirus), helminths (e.g., intestinal trematodes), and protozoans (e.g., *Giardia lamblia*) (52). Some of these bacteria are predicted to carry genes encoding antibiotic resistance, especially if the oyster reef is impacted by sewage-associated inflows (49).

The purpose of this study was to assess the abundance and richness of genes encoding antibiotic resistance in four marine ecosystems along the Texas coastline: Copano Bay, Galveston Bay, Nueces Bay, and 1852 Pass. Each ecosystem is thought to receive a different type of major wastewater inflow dependent on the surrounding land use (e.g., urban, agriculture, industry). This study hypothesized that the dominant bacterial taxa in each sample as well as the abundance and richness of genes encoding antibiotic resistance would reflect the major wastewater inflows. This study also sought to evaluate the potential of the eastern oyster (*C. virginica*) to serve as an indicator species for the detection of ARGs. To date, metagenomic assessments of ARB have rarely been incorporated into water quality monitoring efforts (53, 54).

Data detailing the abundance and richness of ARGs in the marine environment will inform future resource management (55).

CHAPTER II: REVIEW OF THE LITERATURE

The origin of antimicrobial resistance. The phenomenon of antimicrobial resistance is not a modern development. Rather, resistance is an ancient phenomenon that predates the antibiotic era. That is, in ancient environments, bacteria evolved sophisticated allelopathic compounds to cooperate or compete with other microorganisms (16). It is thought that some of these compounds were antimicrobial and thus, the producers of these compounds developed resistance to prevent self-harm and the competitors developed resistance to counter competitive exclusion (56). This ancient history of resistance is evidenced by the discovery of genes coding for ß-lactam and tetracycline resistance in 30,000-year-old permafrost sediments (57). Similarly, the discovery of multidrug resistance in the culturable microbiome of an isolated cave, 4-7 million years removed from surface inputs, further demonstrates that the natural environment is a reservoir for genes encoding resistance (58).

Antibiotics and their mechanisms of action. Since the discovery of penicillin by Alexander Fleming in 1928, many antibiotic compounds have been discovered or synthesized for the treatment of bacterial infections (59). Major classes of antibiotics include the β-lactam antibiotics, macrolides, aminoglycosides, fluoroquinolones, rifamycins, oxazolidinones, lincosamides, sulfonamides, streptogramins, trimethoprims, tetracyclines, phenicols, glycopeptide antibiotics, lipopeptide antibiotics, and polypeptide antibiotics (60). While there are many classes of these compounds, most of them affect bacterial cells by at least one of five

mechanisms of action: cell wall synthesis inhibition, nucleic acid synthesis inhibition, protein synthesis inhibition, metabolic inhibition, and cell membrane disintegration (6, 60, 61).

The β-lactam antibiotics (e.g., penicillins, monobactams, carbapenems, and cephalosporins) and glycopeptide antibiotics (e.g., vancomycin) inhibit the synthesis of bacterial cell walls (6). Both classes interfere with the formation of peptidoglycan or murein, a highly protective matrix that consists of cross-linked strands of alternating residues of N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) (62). β-lactam antibiotics work to inhibit the enzymes involved in NAM/NAG transpeptidation by binding to them (6, 62). Glycopeptide antibiotics work to inhibit these enzymes by binding to their substrate, the terminal D-alanyl-D-alanine residues of peptidoglycan (62, 63). Both of these actions prevent further peptidoglycan formation, causing its components to accumulate, triggering its destruction by autolysins or hydrolases, which kills the bacterial cell (62).

Fluoroquinolones (e.g., ciprofloxacin) and rifamycins (e.g., rifampicin) inhibit the synthesis of bacterial nucleic acids. Fluoroquinolones inhibit the actions of DNA gyrase (which produces tension-relieving negative supercoils during DNA replication) and topoisomerase IV (which has the same function as DNA gyrase, but also decatenates replicated DNA) (64). Rifamycins work to inhibit the β-subunit of bacterial RNA polymerase, preventing transcription (65). Ultimately, the actions of these antibiotics result in double-strand breaks in the bacterial DNA, halting of essential protein production and leading to cell death (6).

Macrolides (e.g., erythromycin), aminoglycosides (e.g., kanamycin), oxazolidinones (e.g., linezolid), lincosamides (e.g., clindamycin), streptogramins (e.g., quinupristin), tetracyclines (e.g., doxycycline), phenicols (e.g., chloramphenicol), and pleuromutilins (e.g., retapamulin) all inhibit the synthesis of bacterial proteins (6, 60). They accomplish this by

binding to either the 30S subunit (comprised of 21 proteins and 16S rRNA) or the 50S subunit (comprised of 36 proteins, 5S rRNA, and 23S rRNA) of the 70S bacterial ribosome (66). Aminoglycosides and tetracyclines bind to the 30S subunit of the ribosome (tetracycline binding is reversible) (67, 68). Macrolides, lincosamides, oxazolidinones, streptogramins, phenicols, and pleuromutilins bind to the 50S subunit of the ribosome (macrolide, lincosamide, and phenicol binding is reversible) (69-72). These antibiotics bind to multiple sites on each of the ribosomal subunits, working to interfere with the binding of translational elements during protein formation, functioning as either a bacteriostat or bactericide (67-72).

Sulfonamides (e.g., sulfamethoxazole) and trimethoprims (e.g., tetroxoprim) inhibit bacterial folate synthesis (BFS), which is necessary for cell growth and DNA synthesis (6, 73). Sulfonamides block the binding of dihydropteroate synthase to p-aminobenzoic acid (pABA; a folate precursor), preventing the formation of dihydropteroate, an essential intermediate compound in BFS (73, 74). Trimethoprims inhibit BFS by preventing a subsequent step of this process; these antibiotics bind to dihydrofolate reductase, which prevents the formation of tetrahydrofolate (involved in the synthesis of nucleobases and amino acids) from dihydrofolate, produced from glutamic acid and dihydropteroate (73, 75, 76). These bacteriostatic antibiotics are often used together, which renders their actions bactericidal (77).

Lipopeptide and polypeptide antibiotics (e.g., daptomycin and polymyxin B, respectively) are bactericidal compounds that disrupt or disintegrate bacterial cell membranes (6). It is hypothesized that the actions of some lipopeptides can cause the leakage of ions from the bacterial cell, effectively depolarizing the cell membrane and rendering multiple cellular systems fatally dysfunctional (6, 78). Other lipopeptides can act as surfactants, permeabilizing the bacterial cell membrane and causing leakage of cellular contents or disintegrating the cell

membrane entirely (79). Polypeptide antibiotics have very similar effects on bacterial cytoplasmic and outer membranes (6, 80, 81). Many of these peptide antibiotics are excessively toxic to humans and are generally only administered topically (81).

The rise of resistance. Since the establishment of penicillin and other β-lactam antibiotics as treatments for bacterial infections in the 20th century, many types of ARB have been discovered. Pathogenic members of the family *Enterobacteriaceae* have become resistant to β-lactam antibiotics; these bacteria are known as the carbapenem-resistant *Enterobacteriaceae* (CRE) (4). *K. pneumoniae* and *E. coli*, two major etiologic agents of pneumonia and diarrhea/dysentery, respectively, are CRE of particular concern (82, 83). Methicillin-resistant *S. aureus* (MRSA) is widespread, resistant to many antibiotic compounds, and a common cause of nosocomial infections. Since the discovery of MRSA in the 1960s, strains have become increasingly resistant to glycopeptides, a major concern since glycopeptides are often the only treatment option for certain MRSA infections (84). Vancomycin-resistant enterococci (VRE), which can be resistant to β-lactam antibiotics, aminoglycosides, and other compounds, were described by Uttley et al. in 1988 (85, 86). These bacteria are major etiologic agents of bacteremia, gastroenteritis, urinary tract infections, and bacterial endocarditis (87).

The appearance of MDR bacteria and extensively drug-resistant bacterial pathogens is especially concerning. The CDC reports that each year, MDR *A. baumannii* and *Pseudomonas aeruginosa* cause 14,000 infections in the U.S., resulting in nearly 1,000 deaths (5). *Mycobacterium tuberculosis*, the etiologic agent of tuberculosis (TB), infected over 9 million people globally in 2006, resulting in nearly 2 million deaths (88). Extensively drug-resistant *M. tuberculosis* (XDR-TB) is characterized by resistance to rifampin and isoniazid (two of the first-line drugs of treatment for TB), a fluoroquinolone, and at least one of the following second-line

drugs: kanamycin, amikacin, or capreomycin (88-90). Infections by XDR-TB are uncommon in the U.S., but cases have been documented on every continent. In South Africa, the high mortality rates of 33% and 98% have been reported for immunocompetent and immunodeficient patients infected with XDR-TB, respectively (89, 91). Similarly, strains of extensively drug-resistant *A. baumannii* (XDR-AB), a pathogen that causes nosocomial infections, have been discovered. Recently, a woman in the U.S. contracted a nosocomial infection caused by a strain of *A. baumannii*. This isolate was found to be resistant to every antibiotic drug tested except colistin, often referred to as the "drug of last resort" (92, 93). Following unsuccessful courses of treatment with colistin, tigecycline, and cefepime, a second *A. baumannii* isolate was recovered from the patient that was resistant to every antibiotic drug available (92).

The administration of antibiotics to both humans and animals has become widespread since their discovery, as they have proven to be important life-saving compounds, effectively extending the human life expectancy (2). The production and consumption of these compounds continues to increase. Between 2000 and 2010, data from 71 countries indicated that human consumption of antibiotics increased by 36% (94). However, the amount of antibiotics consumed by animal agriculture is far greater, accounting for approximately 80% of consumption in the U.S. (2, 95). In 2010, the global use of antibiotics in the raising of livestock exceeded 63,000 tons and is expected to exceed 105,000 tons by 2030 (96). While antibiotics are used for the treatment of infections, they are often administered to livestock and other food animals in sub-therapeutic doses to enhance growth (97). This practice has been linked to the emergence of new, more virulent and more resistant bacteria (98) .

It is this continued exposure to unnatural levels of antibiotics that selects for the enrichment and maintenance of resistant bacteria. Hospitals, nursing homes, and daycare

facilities are among the best examples of environments where a continuous exposure to high-levels of antibiotics has selected for the enrichment of resistant bacteria. Of course, the risk of nosocomial infection is compounded by admission of patients infected with a resistant bacterium. A study of 80,089 U.S. hospital infections revealed an alarming incidence of vancomycin-resistant *Enterococcus faecium* (87.1% of 4,024 isolates) and methicillin-resistant *Staphylococcus aureus* (56.8% of 23,477 isolates) (99). Similarly, in animal agriculture, concentrated animal feeding operations (CAFOs) are hotspots for the enrichment and maintenance of resistant bacteria (100).

The dynamic nature of resistance. Resistance can be intrinsic or acquired. The former refers to a bacterium's innate resistance to a particular antimicrobial agent, which can be attributed to a variety of mechanisms including: 1) a negligible affinity between the antimicrobial and its cellular target; 2) the inability of the antimicrobial to enter the bacterium; 3) the presence of active transporters (e.g., efflux pumps) capable of exporting the antimicrobial; or 4) the production of enzymes (e.g., ß-lactamases) that destroy the antimicrobial. For example, Gram-positive bacteria are generally intrinsically resistant to ß-lactam antibiotics, such as penicillins, due to various modifications of the bacterium's penicillin binding proteins (3). The latter refers to the acquisition of resistance due to mutation or the acquisition of resistance genes via other organisms in a process called horizontal gene transfer (HGT) (101). For instance, some bacteria have acquired macrolide resistance due to mutation of the 23S rRNA, resulting in structural changes in ribosomal macrolide binding sites (102). However, these two examples only touch upon the diversity and complexity of intrinsic and acquired resistance, as recent studies have shown that resistance can involve many elements, which collectively comprise a bacterium's resistome (103).

The presence of antimicrobial compounds can promote the acquisition or evolution of resistance through spontaneous mutation or horizontal gene transfer (104). Mutation is a deceivingly powerful means of acquiring resistance in which a spontaneous nucleotide change that decreases susceptibility will be preferentially passed on to the next generation in a process known as natural selection. Laboratory-based studies have recently shown than mutation alone, over the course of a ten-day experiment, will allow a susceptible strain to evolve resistance to a dose of trimethoprim that is 1,000 times higher than the dose that killed its progenitors (105).

Horizontal gene transfer (HGT) can speed the acquisition of resistance. This is the process by which genetic material is transferred horizontally between bacteria by one of three mechanisms: transformation, transduction, or conjugation (101). Transformation involves the uptake of exogenous DNA by the cell, transduction involves the introduction of foreign DNA by a bacteriophage, and conjugation involves the transfer of foreign DNA through cell-to-cell contact via a conjugation pilus (106). Transposons, plasmids, insertion sequences, integrons, and pathogenicity islands are examples of large pieces of transferrable DNA known as mobile genetic elements or MGEs (13). These MGEs frequently carry resistance genes and their transfer plays a critically role in the spread of resistance.

Proximity is critical for efficient HGT. It follows that biofilms, where high densities of bacteria coexist, are an ideal environment for gene transfer. The frequency of HGT (and, consequently, ARG transfer) is correlated with bacterial density, a factor significantly affected by substrate type (107, 108). For example, in aquatic environments, biotic and abiotic substrates can support bacterial densities two to three orders of magnitude higher than the surrounding water column (108).

Mechanisms of resistance. In general, resistance can be conveyed through five cellular mechanisms: antibiotic target/receptor modification, gene mutation, cell wall or cell membrane permeability modifications, antibiotic compound inactivation, and efflux pump ejection of the antibiotic compound (13, 61). Bacterial resistance against β-lactam antibiotics, glycopeptides, MLSK antibiotics (antibiotics in the macrolide, lincosamide, streptogramin, and ketolide classes), tetracyclines, fluoroquinolones, trimethoprims, and sulfonamides can be conferred by antibiotic target/receptor modifications. Penicillin-binding protein (PBP) and peptidoglycan alterations (coded for by genes such as *mecA* and *vanA*) can provide bacteria with resistance to β-lactam antibiotics and glycopeptides, which inhibit cell wall synthesis (3, 109). Resistance against MLSK antibiotics and tetracyclines, coded for by genes such as *erm* (which is involved in ribosomal RNA methylation) and *tetO* (similar to guanosine 5'-triphosphate-binding proteins, which play a role in protein synthesis and signal transduction pathways), involves protection/modification of the ribosomes, which are the targets of these drugs (3, 110). Different forms of genes such as *qnr* prevent fluoroquinolones from inhibiting the actions of topoisomerase IV and DNA gyrase, important elements in bacterial DNA replication and transcription that are targeted by these antibiotics (3, 111). Trimethoprims and sulfonamides, which inhibit the actions of dihydrofolate reductase and dihydropteroate synthase (necessary for nucleic acid synthesis and cell growth), are also made ineffective by antibiotic target modifications (e.g., genes *dfr* and *sul*) (3, 112). In resistant strains, these enzymes are modified and cannot be bound by these drugs, allowing nucleic acid synthesis in targeted cells to continue unabated (61).

Mutations in bacterial chromosomes can result in resistance to antibiotics such as rifamycins, fluoroquinolones, trimethoprims, sulfonamides, tetracyclines, aminoglycosides,

MLSK antibiotics, oxazolidinones, and lipopeptides. Mutations in *rpoB*, the RNA polymerase target of rifamycins, prevent the binding of this antibiotic, which normally functions to halt transcription (3, 113). Similarly, fluoroquinolone, trimethoprim, and sulfonamide resistance can often be acquired by mutations in genes *gyr/par*, *dfr*, and *sul*, respectively, which are involved in DNA replication and transcription (3, 114). Less common are gene mutations in ribosomes, the targets of tetracyclines and aminoglycosides (e.g., *rrs*, *rrn*, and *rpsL* mutation-conferred resistance), MLSK antibiotics (e.g., *rrl* and *rpl* mutation-conferred resistance), and oxazolidinones (e.g., *rrl* and *rrn* mutation-conferred resistance) (3, 115, 116). Lipopeptide antibiotics, such as daptomycin, target bacterial cell membranes, but can be ineffective against bacteria with mutations in genes such as *rpoB* and *mprF* (3, 117, 118).

The modification of bacterial cell wall and cell membrane permeability also confers resistance to antibiotics. The outer membrane (OM) of Gram-negative bacteria is complex and contains many different types of proteins, including those that are involved in the transport of macromolecules into and out of the cell. The core oligosaccharide component of the OM lipopolysaccharide (LPS) layer can provide Gram-negative bacteria with lipid-mediated resistance against rifamycins, macrolides, aminoglycosides, and other hydrophobic antibiotic compounds (119). However, this resistance can be significantly reduced or eliminated by membrane permeabilizers such as polymyxin B (120). Alteration or lack of certain OM porin proteins, such as OmpF, provide Gram-negative bacteria with resistance against hydrophilic antibiotics, such as β-lactam antibiotics, fluoroquinolones, chloramphenicol, and tetracyclines (119). Conversely, in Gram-positive bacteria such as *S. aureus*, thickening of the bacterial cell wall can confer resistance to aminoglycosides and glycopeptide antibiotics, such as amikacin and vancomycin, respectively (121, 122).

Bacterial enzymes that modify and inactivate antibiotic compounds are numerous and can effectively provide bacteria with resistance against β-lactam antibiotics, chloramphenicol, tetracyclines, MLSK antibiotics, and aminoglycosides. β-lactamases (which hydrolyze penicillins, cephalosporins, and carbapenems) are likely the most numerous of these enzymes and have been traditionally divided into four classes: A, B, C, and D, which either have an active site serine residue (classes A, C, and D) or are metalloenzymes with zinc cofactors (class B) (3, 123). Chloramphenicol, a bacteriostatic antibiotic that inhibits protein synthesis, is inhibited by chloramphenicol acetyltransferase, which is encoded by the *cat* gene (124). Chloramphenicol acetyltransferase production is the primary bacterial mechanism of resistance to this drug (3). Tetracyclines, also inhibitors of protein synthesis, are targeted by the TetX monooxygenase (3). This enzyme is most notably effective against tigecycline, a powerful, newly developed antibiotic that has proven to be effective against multidrug-resistant strains (125). Many nucleotidyltransferases, acetyltransferases, and phosphotransferases can alter aminoglycosides; the genes coding for these enzymes (e.g., *ant, aac,* and *aph*) are widespread and are largely borne on MGEs (3, 126). Similarly, the genes coding for the enzymes that modify MLSK antibiotics are commonly found on MGEs (3, 127-130).

The ejection of antibiotic compounds by the actions of efflux pumps is a broad and highly effective means of resistance. There are five families of efflux pump proteins: the adenosine triphosphate (ATP)-binding cassette (ABC) transporter family, the multidrug and toxic compound extrusion (MATE) family, the resistance-nodulation-division (RND) family, the small multidrug resistance (SMR) family, and the major facilitator superfamily (MFS) (131, 132). In the context of antibiotic ejection, these efflux pumps can be specific (transport one class of antibiotics) or broad (transport multiple classes of antibiotics), the latter often contributing to

multidrug resistance (MDR) (133, 134). Efflux pumps provide pathogens with resistance to many different antibiotics. For example, efflux pumps grant pathogens such as *P. aeruginosa* (an etiologic agent of pneumonia and other infections) with resistance to β-lactam antibiotics, tetracyclines, fluoroquinolones, and chloramphenicol (e.g., the MexCD-OprJ, MexXY-OprM, MexEF-OprN, and MexAB-OprM pumps) (131, 135).

Resistance in aquatic environments. Antibiotic resistance has existed in nature for millennia, but the recent increase in use of antibiotics has resulted in the emergence of new drug-resistant strains at a growing rate (136). This rise of resistant bacteria is well documented in built environments, but an increasing prevalence of resistant environmental bacteria may signal an emerging environmental crisis, rendering some aquatic and marine bacterial diseases more severe or lethal. For example, a recent study found a higher-than-expected resistance to sulfonamides (70%), amoxicillin (12%), and trimethoprim-doxycycline (10%) (137) among 50 environmental *V. cholerae* isolates from coastal Haiti (133). The mechanisms selecting for this resistance were unclear, but it is hypothesized that environmental pollutants linked to resistance were to blame.

The presence of residual antimicrobial compounds is expected to be greatest in proximity to human population centers. Bays, estuaries, and other water bodies adjacent to urban areas are sinks for anthropogenic wastewater and sewage, which often contain bacterial pathogens, residual antibiotics, other pharmaceuticals, heavy metals, halogenated pollutants, and other toxic compounds (16, 22, 23, 56). Thus, coastal ecosystems are confluxes of natural and sewage-associated bacteria and a range of chemical compounds (16, 138). The presence of these compounds may select for the maintenance of resistance in the natural environment (12, 16, 56, 139, 140). Further, the co-existence of anthropogenic bacteria and environmental bacteria creates

an ideal scenario in which the close proximity of these bacteria could promote the horizontal transfer of resistance genes to natural bacterial populations, leading to the evolution of newly resistant pathogens (56).

The accumulation of chemical compounds that select for resistance in natural environments is a recent discovery. However, it is now well documented that residual antibiotic compounds and a variety of chemical pollutants select for the spread and maintenance of ARGs (12, 139, 140). These residual compounds are typically released into natural environments through freshwater inflows. For example, triclosan is a mass-produced antimicrobial compound found in sanitary soaps, but WWTPs cannot effectively remove triclosan or similar compounds (24). In the U.S., an estimated 1.1 to 4.2 x 10^5 kg of triclosan is discharged into the environment every year (141). Although the effects of triclosan on environmental bacteria are largely unknown, triclosan has been linked to the development of multidrug resistance in pure cultures (141, 142). Regardless, the potential risks associated with triclosan prompted the Food and Drug Administration to recently ban the use of triclosan and 18 other antimicrobial chemicals in hand soaps and body washes (143).

Legislative measures like the FDA's ban on triclosan represent a positive step toward limiting the rise of antibiotic resistance in the environment. However, a larger source of antimicrobial runoff remains largely unregulated, as antibiotics are used more heavily in animal agriculture (144). Runoff from intensive animal agriculture (e.g., cattle, swine, and poultry) has been shown to load groundwater and surface water with residual antimicrobials (145, 146). Even the amendment of farm soils with manure fertilizer increases the prevalence of ARB, which wash into aquatic environments during storm events (147, 148). The lack of direct non-point

source regulation stems from the aim of the Clean Water Act, which specifically targets point source water pollution (149).

Heavy metals are yet another contaminant known to contribute to the development of multidrug resistance. A recent study revealed that sub-lethal concentrations of heavy metals select for the maintenance of a 220 kb extended-spectrum, β-lactamase plasmid in *E. coli* (150). This indirect development of resistance is due to 1) the frequent genetic linkage between genes that encode heavy metal resistance and antibiotic resistance, which can be located on the same MGE and 2) the dual-purpose nature of multidrug efflux pumps, which detoxify and protect the cell from a range of toxins including antibiotics. In either case, exposure to heavy metals would co-select for metal resistance and antibiotic resistance. For example, a novel efflux pump was previously identified in *Serratia marcescens* that was isolated from a stream impacted by heavy metal contamination (151).

The emerging environmental crisis. Historically, environmental bacteria, such as *Vibrio* species, have been generally susceptible to treatment with antibiotics, but a growing number of studies are reporting the incidence of environmentally acquired infections that are resistant to one or more antibiotics (152, 153). The prospect of resistant *Vibrio* species is especially alarming given that vibriosis affects an estimated 85,000 persons per year in the U.S. (154). More alarming is the prospect of antibiotic-resistant cholera, which is contracted by over 2.8 million persons per year worldwide (155). However, the impact of antimicrobial pollution in the marine environment remains largely unknown.

It is possible that residual antimicrobial compounds, present at low concentrations in the coastal marine environment, have selected for this recent rise in resistance. It is also possible that residual antimicrobials could affect environmental microbes in unpredictable ways. For example,

a microcosm-based study reported that minute concentrations of triclosan (≈ 5 ppm) in seawater induce exponential (68 – 1,700 fold) *Vibrio* growth (156). Thus, to counter this emerging environmental crisis, surveillance and risk assessment procedures, analogous to those implemented in hospitals, are needed to assess and manage the risks of antibiotic resistance in the environment. In this study, procedures were developed to assess the prevalence of ARGs in water and oysters collected from the marine environment.

CHAPTER III: METHODOLOGY

Study sites. Samples were collected from four sites along the Texas coast: Copano Bay (28.117752, -97.156441), Galveston Bay (29.255072, -94.917916), Nueces Bay (27.877019, -97.504845), and 1852 Pass (27.625230, -97.208699). (Figure 1).

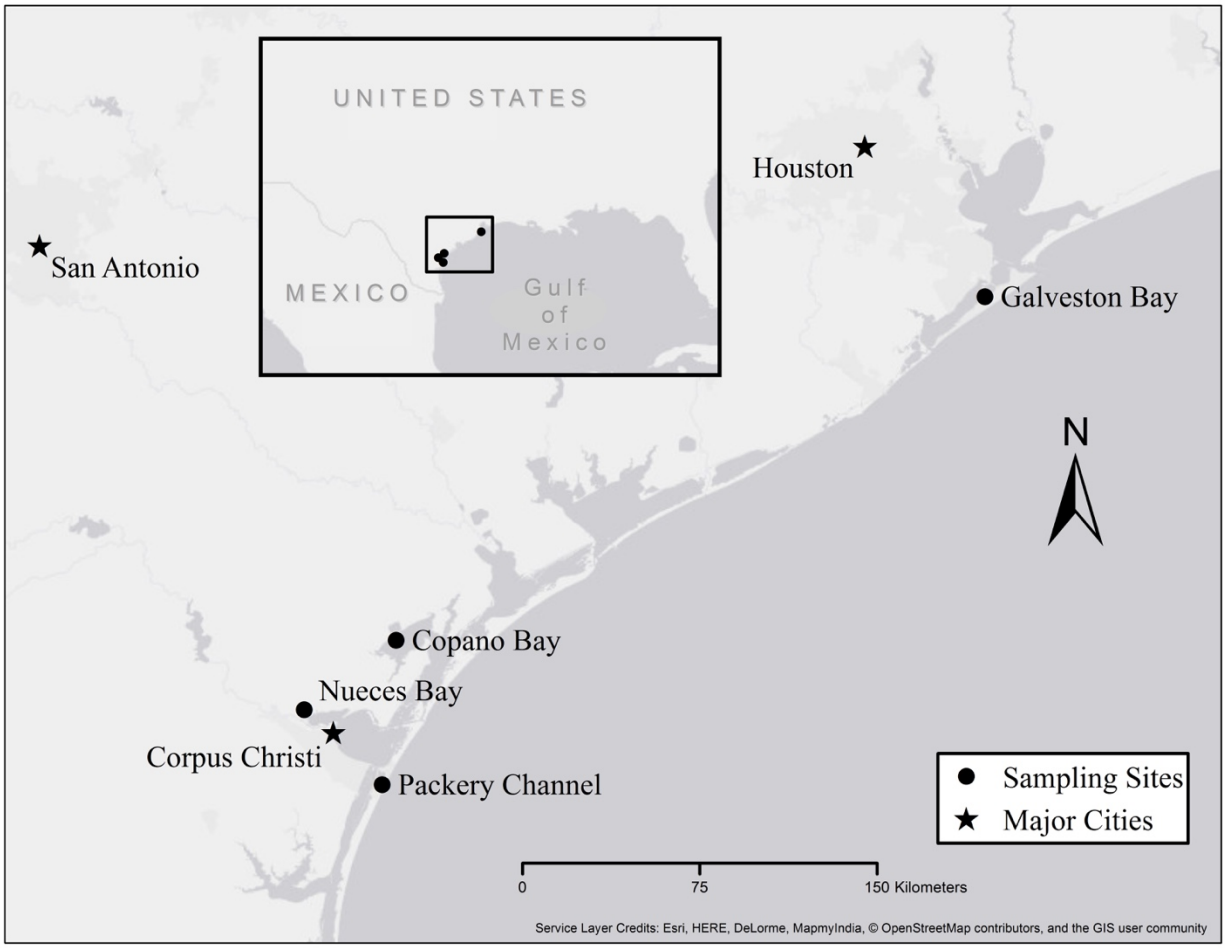

Figure 1. Map of the oyster and water sample collection sites in four Texas marine ecosystems: Copano Bay, Galveston Bay, Nueces Bay, Packery Channel (1852 Pass).

Copano Bay, Texas, is part of the Mission-Aransas Estuary and covers approximately 463 km^2, averages 2 m in depth, and has an average water residence time of 3 years (157). The

estuary receives an average of approximately 0.6 km^3 of freshwater inflow each year (158). The bay receives freshwater from three major sources: Copano Creek, the Aransas River, and the Mission River, which discharge approximately 1.3 m^3, 1.2 m^3, and 4.5 m^3 of freshwater per second, respectively (159). On average, approximately 0.72 km^3 of the Mission-Aransas Estuary's water is lost due to evaporation each year, resulting in an average total freshwater inflow balance of approximately 0.35 km^3 (158). The watersheds of the Mission and Aransas rivers cover approximately 2,675 km^2 and 2,146 km^2, respectively, and much of this area is agricultural or scrubland (157).

Galveston Bay, Texas, part of the Trinity-San Jacinto Estuary, is the Gulf of Mexico's second largest estuary and covers approximately 1,360 km^2 with an average depth of 2 m (160). It receives an average of approximately 13.9 km^3 of freshwater inflow each year, of which 83% is supplied by Buffalo Bayou and the Trinity and San Jacinto rivers (161). Approximately 60% of the wastewater discharge of Texas flows into Galveston Bay (www.tamug.edu/gbic/gbicresources/bayfacts.html). On average, approximately 1.1 m of the bay's water level is lost due to evaporation each year (162). The total freshwater inflow balance after considering evaporation averages approximately 13.8 km^3 annually (163). The Galveston Bay watershed spans approximately 38,600 km^2 and contains many urban areas (including Houston, the most populous Texas city) and over half of the Texas population (www.gbep.state.tx.us/discover-galveston-bay/). The land surrounding this bay is largely urban or industrial (www.gbep.state.tx.us/discover-galveston-bay/). Additionally, oysters harvested from Galveston Bay constitute over half of the state's total oyster harvest (164).

Nueces Bay, Texas, part of the Nueces Estuary, covers approximately 75 km^2 and has an average depth of 0.7 m (165). The estuary receives an average of approximately 0.72 km^3 of

freshwater inflow each year (166). On average, approximately 0.83 km^3 of water is lost due to evaporation each year, resulting in an average total freshwater inflow balance of approximately 0.32 km^3 (166). In 1982, the erection of Choke Canyon Dam altered the quantity and timing of freshwater entering the estuary from Nueces River (Nueces Bay's primary source of freshwater), converting it from a positive estuary to a negative estuary (167). Additionally, Nueces Bay is proximal to many petroleum and gas fields and served as a petroleum brine discharge location from 1900 to 1993 (168). Petroleum brine has been found to contain many different types of polycyclic aromatic hydrocarbons (PAHs), compounds that are known to enrich ARGs in the environment (169). Sediment, sediment pore water, and fauna (namely birds and oysters) recovered from Nueces Bay have been found to contain high levels of toxic compounds, such as heavy metals and PAHs, as a result of exposure to these petrochemicals (170-172).

The 1852 Pass site is immediately adjacent to Packery Channel, an artificial inlet that connects the Gulf of Mexico to the upper Laguna Madre that is approximately 37 m wide, 5.6 km long, and 4 m in depth (173). The inlet directly exchanges water with the Gulf of Mexico. Save for a small residential community, it is surrounded by largely undeveloped coastal wetland.

Sample collection. All sites were sampled once over a 7-week period in 2016. Galveston Bay was sampled on June 21, Copano Bay was sampled on July 7, 1852 Pass was sampled on July 21, and Nueces Bay was sampled on July 27. During each sampling event, twelve market-sized (≥ 76 mm) oysters (*C. virginica*) and 1 L of ambient water (taken from a depth of 0.5 m) was collected from a single oyster reef at each site. The collection of twelve oysters accounted for biological variation as recommended by the US Food and Drug Administration (FDA) Bacteriological Analytical Manual (BAM) (174). Each 1 L ambient water sample was collected using an autoclave-sterilized polypropylene bottle. Environmental conditions (water temperature,

conductivity, dissolved oxygen, and pH) were recorded with a YSI 556 Multiprobe (YSI Inc., Yellow Springs, OH). Samples were stored on ice (at approximately 4ºC) during the 4-hour transport to the laboratory at Texas A&M University-Corpus Christi (TAMU-CC). Upon arrival, samples were held in a refrigerator at 4°C. All samples were processed within 24 hours of sample collection.

Oyster sample processing. Oysters were washed in 70% ethanol and excess sediment, barnacles, and other debris were removed from the exterior with a sterile oyster knife. Oyster measurements (shell length, wet body mass, and pooled stomach content mass) were reported in Table S1. The oysters were then shucked and the stomach was removed aseptically using a flame-sterilized razor blade. Approximately 0.5 cm^3 of stomach contents were transferred to a 50 mL centrifuge tube containing 8 mL of phosphate-buffered saline (PBS). To account for natural variation in stomach contents and ensure rapid processing, the 12 stomach extracts were pooled into groups of four to yield three composite samples. Bacteria were isolated from the pooled samples by serial filtration and centrifugation as described previously (175). This step was taken to exclude as many oyster cells and other debris from the samples as possible. Briefly, pooled stomach contents were vortexed on medium speed with pulsing for 30 seconds. The contents were then allowed to sediment by gravity before the supernatant was collected and transferred to a new 50 mL centrifuge tube. The supernatants were serially vacuum-filtered using 100 μm and then 20 μm Steriflip® Filter Units (EMD Millipore, Billerica, Massachusetts, USA). The filtrates were centrifuged twice at 300 x g for 15 minutes at room temperature to precipitate eukaryotic cells and other debris. The filtrates were then centrifuged at 9,168 x g for 15 minutes at room temperature to pellet bacterial cells. The supernatants were discarded and the bacterial pellets

were resuspended in 1 mL of PBS, transferred to 2 mL microcentrifuge tubes, and washed twice with PBS at 9,168 x g for 15 minutes at room temperature.

Water sample processing. Water samples (1 L) were prefiltered through 315 μm nylon mesh to remove larger debris and organisms before serial filtration through 100 μm and 20 μm Steriflip® Filter Units (EMD Millipore, Billerica, MA). The bacteria present in a 100 mL aliquot of filtrate were collected by membrane filtration on a 0.22 μm polycarbonate filter (47 mm in diameter). This process was repeated three times. The three filters were then cut into 0.5 cm^2 pieces with a flame-sterilized scalpel and placed in separate 2 mL microcentrifuge tubes containing 1 mL of PBS.

DNA isolation. DNA was isolated from six samples (3 pooled oyster samples and 3 water samples) per study site (N = 4) for a total of 24 samples. Total genomic DNA was isolated from these environmental samples using a modified sodium dodecyl sulfate (SDS)-based extraction method originally developed to recover genomic DNA from soils (176). Briefly, samples were resuspended in 540 μL of extraction buffer (EB) (100 mM Tris-HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, 1% CTAB) with 2 μL of proteinase K solution (20 mg/mL) and incubated at 37°C for 30 minutes with horizontal shaking at 350 rpm. Following this incubation, 60 μL of 20% SDS was added before incubating at 65°C for two hours with gentle end-over-end inversion every 20 minutes. The supernatants were recovered after centrifugation (6,000 x g for 10 minutes at room temperature) and transferred to new 2 mL microcentrifuge tubes. The pellet was retained and extracted two more times by adding 180 μL of EB and 20 μL of 20% SDS, vortexing for 10 seconds, incubating at 65°C for 10 minutes, and centrifuging at 6,000 x g for 10 minutes at room temperature. The

supernatants from the three rounds of extractions were combined, treated with an equal volume of 24:1 chloroform:isoamyl alcohol (vol/vol), vortexed for 15 seconds, and centrifuged at 6,000 x g for 10 minutes at room temperature. The aqueous phase was recovered and transferred to new 2 mL microcentrifuge tubes. This chloroform:isoamyl treatment, used to remove proteins and polysaccharides, was performed twice and the aqueous phases were combined. Single-stranded RNA was removed by adding 1 μL of RNaseA solution (10 mg/mL) followed by a 37°C incubation for 15 minutes. The genomic DNA was precipitated with 0.6 volume of isopropanol at room temperature for one hour. The tubes were then centrifuged at 16,000 x g for 20 minutes at room temperature to pellet the DNA. The pellets were washed twice with cold (-20°C) 70% ethanol and resuspended in 200 μL of sterile deionized water. The DNA quantity (ng/μL) and quality (260/280 and 260/230 absorbance ratios) were evaluated using a BioPhotometer D30 (Eppendorf AG, Hamburg, Germany) and the final DNA concentrations were verified using a Qubit® Fluorometer (ThermoFisher Scientific, Waltham, MA). DNA was stored in the dark at -20°C prior to sequencing.

Metagenome sequencing. Preparation of True-Seq, PCR-free sequence libraries and metagenome sequencing was carried out by Molecular Research LP (Shallowater, TX). A total of sixteen metagenomes were sequenced: three pooled oyster metagenomes (N = 4 oysters per metagenome) and one pooled ambient water metagenome (N = 3 water samples per metagenome) per study site. Sequencing was carried out on the Illumina HiSeq 2500 platform with 2x150 paired-end read chemistry. To ensure adequate coverage, a full flow cell (up to 4 billion paired reads or 500 Gb) was dedicated to each study site (i.e., 3 oyster metagenomes and 1 water metagenome).

Read pre-processing. The raw DNA sequence reads were submitted to the European Bioinformatics Institute (EBI) Metagenomics Pipeline. The EBI Pipeline includes automated workflows for read processing (i.e., merging paired reads, quality filtering, and length filtering) (177). Briefly, overlapping reads were merged with SeqPrep (178), low quality bases were removed with trimmomatic (179), and reads less than 100bp in length were removed with a BioPython script.

Taxonomic analysis. The EBI Pipeline also includes automated workflows for taxonomic and functional analysis. Briefly, prokaryotic rRNA reads were filtered with rRNASelector (180) and QIIME (181) was used to annotate the 16S rRNA using the Greengenes reference database (182). The 16S rRNA sequences were input into the Metagenome Analyzer (MEGAN) for microbial community analysis (177, 183). To visualize the composition of each community, the QIIME script "summarize_taxa.py" was used to convert EBI Pipeline output files (BIOM format) into tab-delimited data (181).

ARG identification. A local instance of the Microbial Ecology Group Antibiotic Resistance (MEGARes) database (184) was installed on the TAMU-CC high-performance computer (HPC). The MEGARes database consists of approximately 4,000 hand-curated resistance gene sequences. The pre-processed reads were aligned against the database using the Basic Local Alignment Search Tool (BLASTn) (185). Reads returning alignments covering at least 50bp with an expect value (E-value) cutoff of 10^{-5} (maximum) and a percent identity cutoff of 90% (minimum) were considered positive hits (186, 187). The E-value has a negative relationship with the likelihood that a BLAST hit occurred by chance (i.e., the lower the E value, the more valid the search hit) (188). The oyster sample BLASTn hits were combined for each site, totaling one oyster and one water sample set of hits per site.

ARG diversity and statistical analyses. To determine ARG relative diversity (Shannon-Wiener), evenness, and richness between sites and samples, the vegan Community Ecology package was used in R (189, 190). For statistical analysis of all data, R was used along with the following R packages: multcomp (Multiple Comparisons Using R), car (Companion to Applied Regression), and MuMIn (Multi-Model Inference) (189, 191-193). Additionally, the following source files, developed by Dr. Blair Sterba-Boatwright (TAMU-CC), were used: augPairs.R, qqnormWithBounds.R, diagPlots.R, traditionalForwardBackward_lm.R, diagPlotsWithoutLMTest.R, and multcompUtilities.R. A one-way ANOVA was used to compare oyster shell length, wet body mass, and pooled stomach content mass between sites. One-way ANOVA was also used to compare ARG abundance and richness between oyster and water samples in general (by sample type). A multiple regression analysis was used to test for relationships between oyster measurements (mean shell length, mean wet body mass, and pooled stomach content mass) (Table S1), DNA yield (Tables S2 and S3) (DNA library sizes are reported in Table S4), ARG abundance and richness, and environmental parameters (temperature, dissolved oxygen concentration, conductivity, and pH). For all statistical tests, significance was declared when the obtained p-value was less than 0.05. A one-way analysis of variance (ANOVA), used to compare oyster shell length, wet body mass, and pooled stomach content mass between sites revealed that these measurements were significantly different between sites. To determine which variables were different between sites, general linear hypothesis tests were then conducted as multiple comparison of means post hoc analyses (Tukey contrasts using the Westfall method).

CHAPTER IV: FINDINGS/RESULTS

Approximately 577 million raw reads were sequenced. On average, approximately 22.1 million and 22.5 million reads were sequenced per oyster and water samples, respectively. Approximately 536 million of these reads were retained following trimming and filtering, totaling 228 GB of sequence data.

Environmental data, oyster measurements, and isolated DNA quantities. During each sampling event, environmental parameters were recorded (Table 1).

Table 1. Environmental parameters of the Copano Bay, Galveston Bay, Nueces Bay, and 1852 Pass sampling sites.

	Copano Bay	Galveston Bay	Nueces Bay	1852 Pass
Sampling Date	7/7/16	6/21/16	7/27/16	7/21/16
Coordinates	28.117752, -97.156441	29.255072, -94.917916	27.877019, -97.504845	27.625230, -97.208699
Temperature (℃)	29.4	31.6	27.7	31.2
DO (%)	91.4	75.8	102.8	81.3
DO (mg/L)	6.68	5.12	7.2	4.91
Conductivity (µS/cm)	13760	28974	33566	63381
pH	8.09	8.22	8.39	8.22

Microbial community analysis. Analysis of the 16S rRNA genes revealed which taxonomic classes were most abundant. The site names were abbreviated as follows: Copano Bay (C), Galveston Bay (G), Nueces Bay (N), and 1852 Pass (P). The sample types were designated as follows: oyster (O) and water (W). A sample name of "CO1" corresponds to the first of three Copano Bay oyster samples. Rarefaction curves of all metagenomes reached an asymptote, indicating raw abundance data can be compared between sites (Figures S1, S2, S3, and S4). This

also indicated that sequencing was deep enough to capture the true diversity of the microbial communities. For all three pooled Copano Bay oyster samples, the highest percentage of sequences belonged to members of the class *Planctomycetia* (Figure 2 and Table 1). This class constituted 73.9, 37.4, and 57.5% of the taxa found in these samples. The Copano Bay water sample was dominated by the class *Acidimicrobiia*, which constituted 21.9% of the community. For all three Galveston oyster samples, the subclass *Synechococcophycideae* (class *Cyanophyceae*) was most abundant, constituting 30, 44.6, and 72.5% of each community. The Galveston Bay water sample was dominated by the class *Gammaproteobacteria* (17.5% of the community). All three Nueces Bay oyster samples were dominated by the subclass *Synechococcophycideae*, constituting 48.9, 44.8, and 48.4% of each community. The Nueces Bay water sample was dominated by the class *Alphaproteobacteria* (23.8% of the community). Two of the 1852 Pass oyster samples were dominated by the class *Mollicutes* (41.2 and 66.5% of each community) while the third was dominated by the class *Gammaproteobacteria* (44.4% of the community). The 1852 Pass water sample was dominated by the class *Alphaproteobacteria* (44.8% of the community).

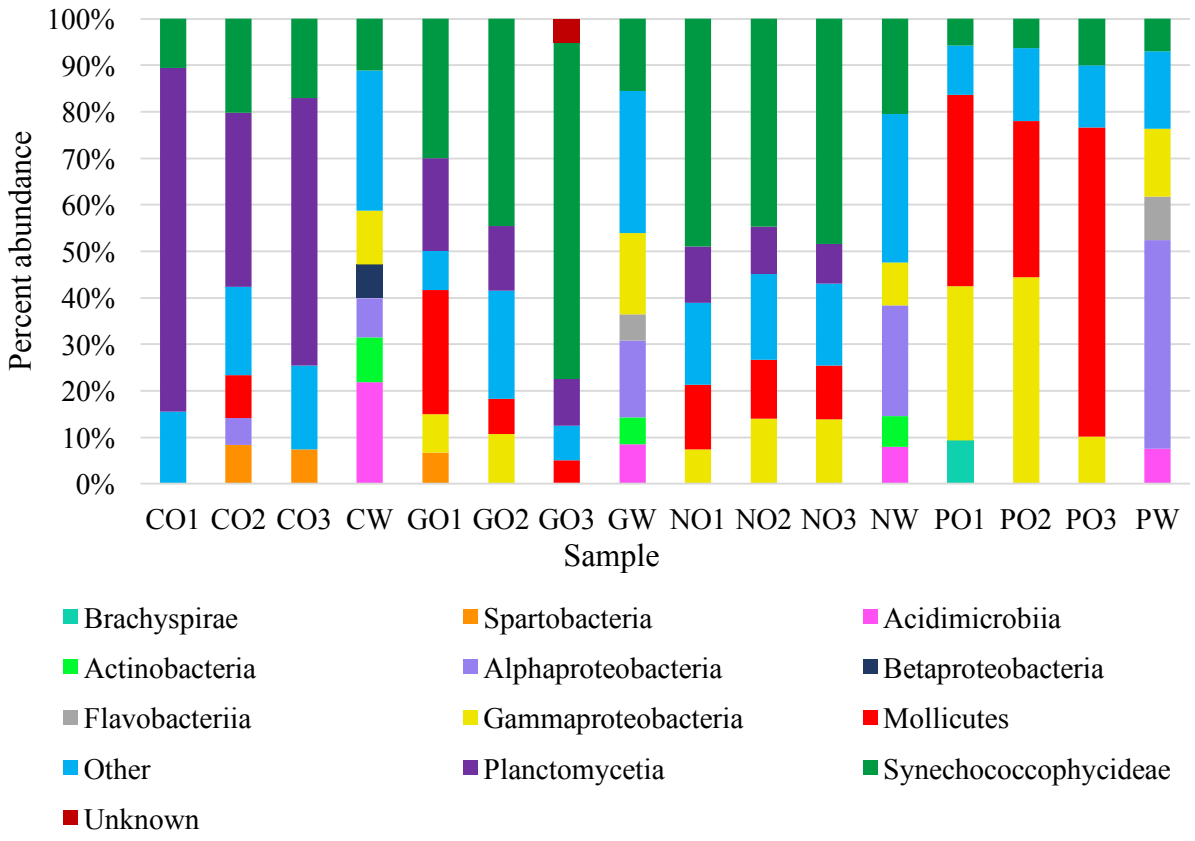

Figure 2. Microbial taxonomy profile of oyster (O) and water samples (W) collected from Copano Bay (C), Galveston Bay (G), Nueces Bay (N), and 1852 Pass (P), Texas. Absolute read counts were used for analysis. Any class that constituted < 5% of a sample's total taxonomic composition was classified as "Other." The vast majority of these Other classes constituted < 1% of these communities.

Table 2. Dominant taxonomic classes in oyster and water samples collected from Copano Bay, Galveston Bay, Nueces Bay, and 1852 Pass, Texas.

	Copano Bay	Galveston Bay	Nueces Bay	1852 Pass
Oysters	PLC	SYN	SYN	MOL GMA
Water	ACD	GMA	APH	APH

PLC = Planctomycetia
SYN = Synechococcophycideae
ACD = Acidimicrobiia
GMA = Gammaproteobacteria
APH = Alphaproteobacteria
MOL = Mollicutes

In two of the three 1852 Pass oyster samples, MOL was the most abundant class. For the rest of the sites, one class was the most abundant in all samples.

Taxonomy-based principal coordinate analysis (PCoA) of the samples revealed that 46.0% and 33.4% of the variation was explained by the first two principal components (Figure 3). Based on this variation (79.4% of the total variation between samples), the water samples clustered together and were distinct from the oyster samples. In contrast, the oysters from Copano Bay, Nueces Bay, and 1852 Pass formed three distinct clusters. Meanwhile, the oysters from Galveston Bay clustered generally with the Nueces Bay oyster samples, but they did not form a distinct cluster.

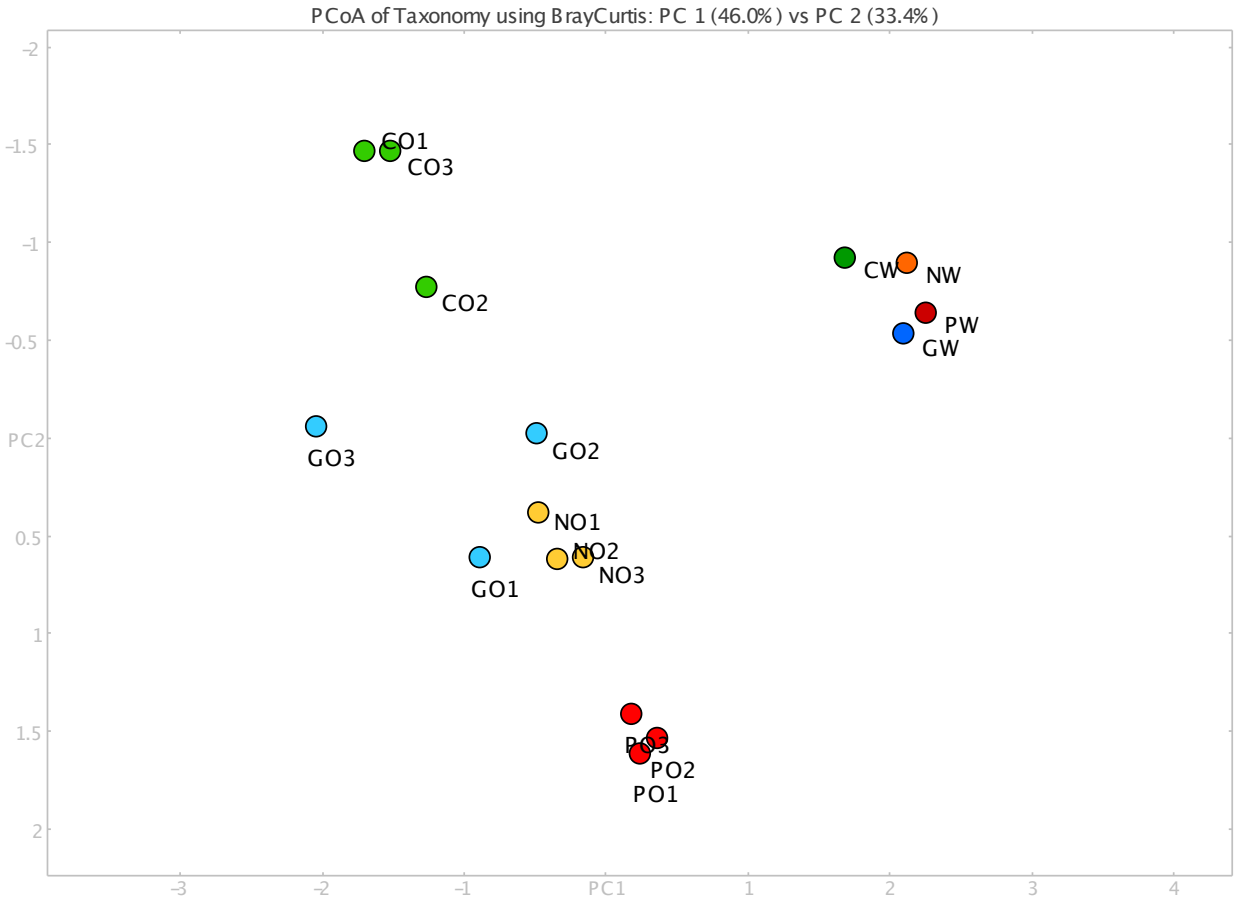

Figure 3. Principal Coordinate Analysis (PCoA) plot showing the taxonomic comparison of oyster (O) and water (W) microbial communities collected from Copano Bay (C), Galveston Bay (G), Nueces Bay (N), and 1852 Pass (P), Texas. The Bray-Curtis dissimilarity statistic was used for this analysis.

ARG identification, enumeration, and diversity. The abundance of ARGs varied widely between samples (Figure 4). Abundances ranged from 28 to 826 genes. The Galveston Bay oyster and water samples each contained the lowest abundance of ARGs for both sample types (28 and 533, respectively). The 1852 Pass oyster samples contained the highest abundance

of ARGs found in oysters (230 ARGs). The Copano Bay water sample contained the highest abundance of ARGs found across all samples (826 ARGs). On average, the water samples contained approximately 7.7 times the number of ARGs as the oyster samples.

In total, 3,516 ARGs comprising 53 ARG types (e.g., *blaTEM*, *tetR*, *mexC*) were identified (Figure 4 and Table S5). ARG richness ranged from 10 to 32 different types of ARGs. The Galveston Bay oyster and water samples each contained the lowest ARG richness for both sample types (10 and 30 types, respectively). Out of all oyster samples, the Copano Bay oyster samples contained the highest ARG richness (17 types). The Nueces Bay and 1852 Pass water samples contained the highest ARG richness out of all water samples and all samples in general (32 types). On average, each site's water sample contained approximately 2.4 times as many types of ARGs (ARG richness) as its oyster samples.

The relative diversity of ARGs varied between samples (Table 2). The oyster samples with the least diverse collection of ARGs were from 1852 Pass, with a Shannon-Wiener (SW) index of 1.49. The Copano Bay oyster samples had the most diverse collection of ARGs of all the samples (SW index of 2.08). The Copano Bay water sample ARGs were the least diverse of all water samples (SW index of 1.83). The Galveston Bay water sample ARGs were the most diverse of all water samples (SW index of 2.03). Pielou's evenness of the Copano Bay water sample and the Galveston Bay oyster sample were the lowest (0.54) and highest (0.87), respectively. On average, the relative SW diversity of ARGs was slightly higher in the water samples (average SW index of 1.92 for water samples, 1.91 for oyster samples).

It was clear which ARGs were the most abundant across samples (Figure 5). The *tuf* gene was the most abundant ARG in the Galveston Bay oyster samples, the Nueces Bay oyster samples, and the 1852 Pass water sample (25.0, 26.2, and 32.0% of all ARGs in these samples,

respectively). The mutated *rpoB* gene was the most abundant ARG in the Copano Bay oyster and water samples (31.0 and 47.0%, respectively), the Galveston Bay water sample (34.7%), and the Nueces Bay water sample (36.1%). The mutated *gyrB* gene was the most abundant ARG in the 1852 Pass oyster sample, constituting 44.8% of the sample's total ARG abundance.

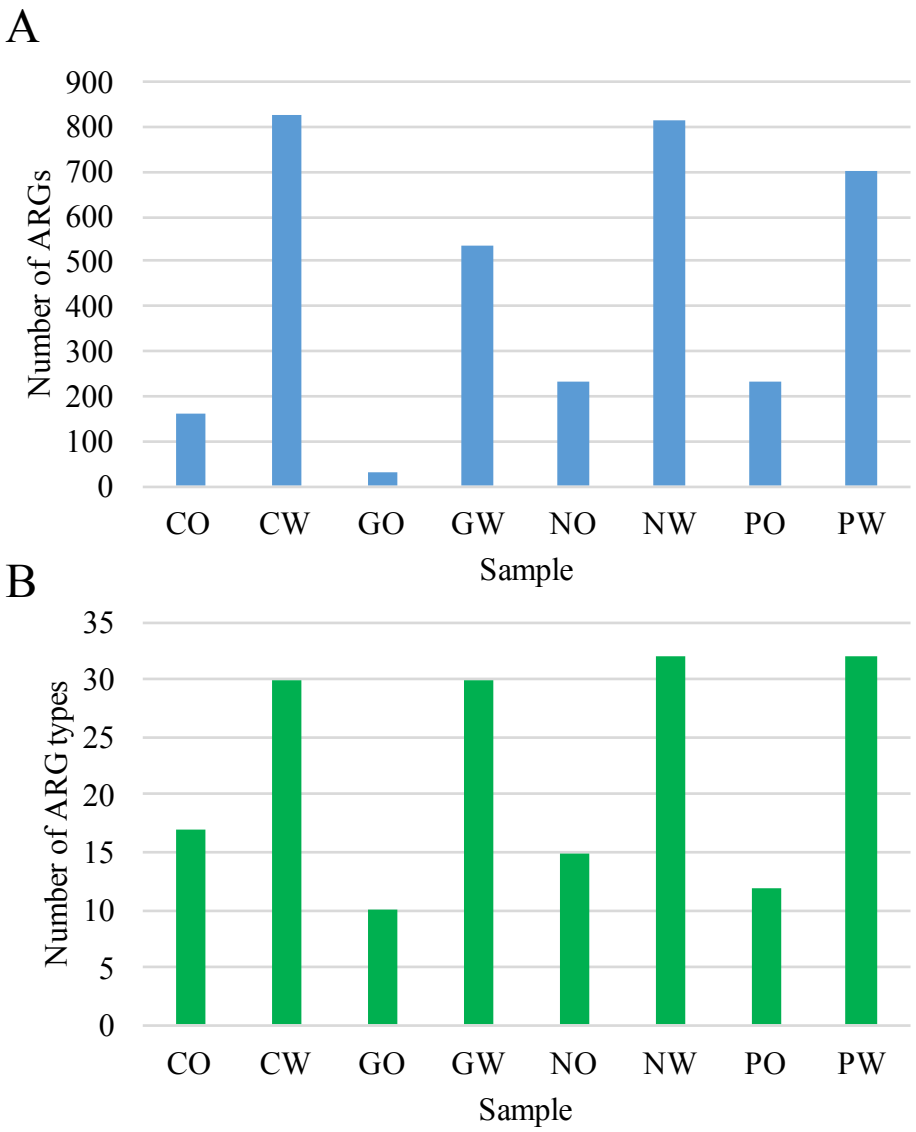

Figure 4. Antibiotic resistance gene (ARG) abundance (A) and richness (B) in oyster (O) and water (W) samples from Copano Bay (C), Galveston Bay (G), Nueces Bay (N), and 1852 Pass (P), Texas.

Table 3. Antibiotic resistance gene (ARG) Shannon-Wiener diversity (SWD), evenness, and richness in oyster (O) and water (W) samples from Copano Bay (C), Galveston Bay (G), Nueces Bay (N), and 1852 Pass (P), Texas.

	CO	CW	GO	GW	NO	NW	PO	PW
SWD	2.0798	1.8336	1.9940	2.0291	2.0712	1.9315	1.4893	1.8832
Evenness	0.7341	0.5391	0.8660	0.5966	0.7648	0.5573	0.5994	0.5434
Richness	17	30	10	30	15	32	12	32

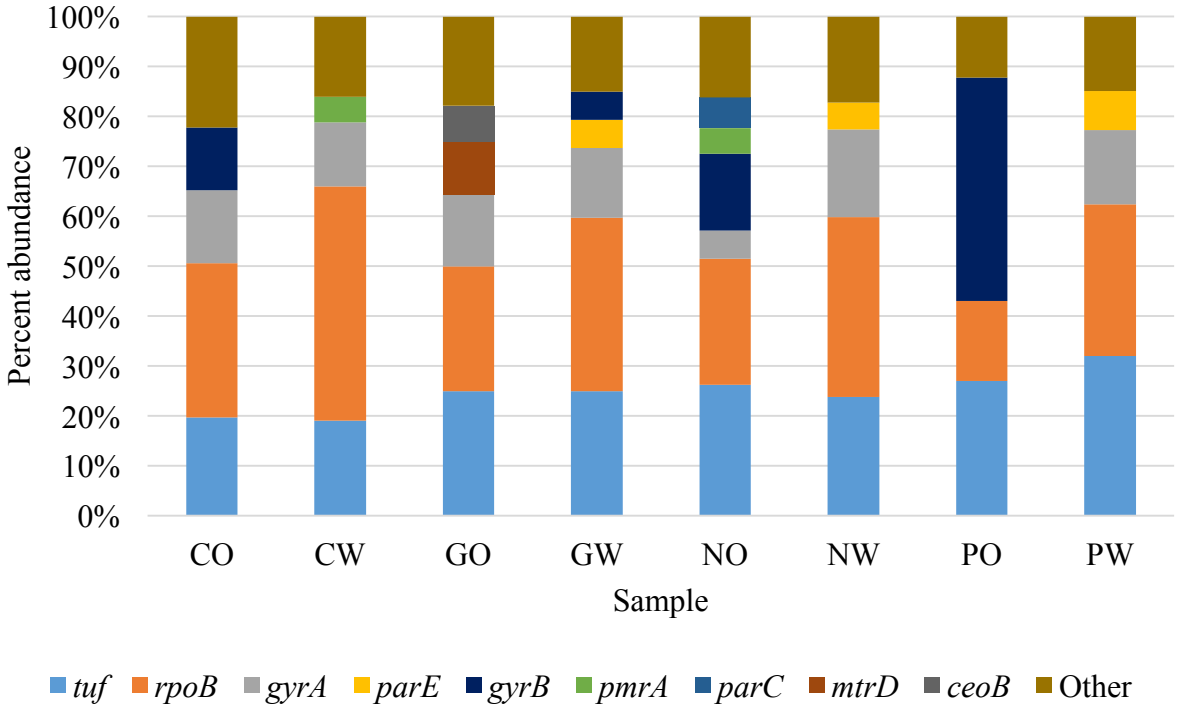

Figure 5. Antibiotic resistance genes (ARGs) and their relative abundances in oyster (O) and water (W) samples from Copano Bay (C), Galveston Bay (G), Nueces Bay (N), and 1852 Pass (P), Texas. Any ARG that constituted < 5% of a sample's total ARG abundance was classified as "Other." The vast majority of these Other ARGs constituted < 1% of these total abundances.

Statistical analyses. These analyses showed that the shell lengths of oysters from Nueces Bay (mean = 105.00 ± 13.89) were significantly larger than the shell lengths of the oysters collected from the other three sites (Copano Bay: mean = 84.67 ± 5.42; Galveston Bay: mean = 85.92 ± 8.03; 1852 Pass: mean = 89.92 ± 8.70). The wet body masses of the Nueces Bay oysters (mean = 17.59 ± 7.04) were also significantly larger than the wet body masses of the oysters collected from the other sites (Copano Bay: mean = 8.70 ± 2.17; Galveston Bay: mean = 12.85 ± 3.69; 1852 Pass: mean = 12.59 ± 3.28). Additionally, the masses of the pooled oyster stomach contents from Copano Bay (mean = 0.92 ± 0.11) were significantly greater than those from 1852 Pass (mean = 0.63 ± 0.06). A one-way ANOVA used to compare ARG abundance and richness by sample type revealed that ARG abundance and richness were significantly greater in the water samples (abundance: mean = 717.75 ± 117.31; richness: mean = 31.00 ± 1.00) than in the oyster samples (abundance: mean = 161.25 ± 82.28; richness: mean = 13.50 ± 2.69).

Multiple regression analysis of DNA yield per sample, ARG abundance and richness data of both oyster and water samples, and environmental data from each site (temperature, dissolved oxygen concentration, conductivity, and pH) revealed a significant positive linear relationship between ARG abundance and richness ($p = 0.00404$, $r^2 = 0.99$) and a negative linear relationship between site temperature and dissolved oxygen concentration ($p = 1.72 \times 10^{-15}$, $r^2 = 1.00$). There was also a significant positive linear relationship between ARG abundance and DNA yield ($p = 0.0281$, $r^2 = 0.92$).

Multiple regression analysis of DNA yield from oyster samples alone, oyster measurements (mean shell length, mean wet body mass, and pooled stomach content mass),

ARG abundance and richness from oysters alone, and environmental parameters from each site (temperature, dissolved oxygen concentration, conductivity, and pH) only revealed a significant positive linear relationship between oyster wet body mass and pH ($p = 0.000776$, $r^2 = 1.00$). Notably, there was no significant relationship between pooled stomach content mass and DNA yield, ARG abundance, or ARG richness.

CHAPTER V: DISCUSSION

The purpose of this study was to assess the abundance and richness of genes encoding antibiotic resistance in four marine ecosystems along the Texas coastline. To accomplish this, bacterial metagenomes were sequenced and analyzed to investigate the abundance and richness of ARGs in oyster and water samples from four sites along the Texas coast. To investigate a possible relationship between dominant bacterial taxa and major wastewater inflow type, which is thought to affect the abundance and richness of ARGs, the microbial community composition of each sample was analyzed. The potential of the eastern oyster (*C. virginica*) to serve as an indicator species for the detection of ARGs was also evaluated.

The usage of land surrounding a body of water affects its hydrochemistry by the inflow of usage-associated chemicals via runoff (such as fertilizer leaching into a lake from nearby farmland). The hydrochemical composition of a body of water can be reliably evaluated by examining the microbial communities residing in the water since a relationship exists between hydrochemistry and microbial community composition. This relationship was used to verify the expected major wastewater inflow type entering each study site.

Planctomycetia was the dominant bacterial class in all three oyster samples from Copano Bay. The phylum *Planctomycetes* contains the bacteria that have been found to utilize anaerobic

ammonium oxidation (termed "anammox") (194, 195). For all other samples, this class was a minority of the community despite the ubiquity of *Planctomycetes* in the marine environment. It is unclear why this was the case. In previous studies, members of this class have been found in large numbers (10^8 cells/mL) in coastal marine sediment (194). The presence of this class can give insight into the hydrochemical conditions of the Copano Bay sampling site, as previous studies have found a link between *Planctomycete* presence and high levels of ammonium nitrate, which is the main component of many agricultural fertilizers (196). These findings suggest Copano Bay receives agricultural runoff, as found in a previous study (197).

Acidimicrobiia was the dominant bacterial class in the Copano Bay water sample. The class *Acidimicrobiia* largely consists of acidophilic bacteria that reduce and oxidize iron compounds (198, 199). Similar to anammox *Planctomycetes*, these bacteria can utilize ammonium oxidation under iron-reducing conditions (termed "feammox") (198, 200). The abundance of this class in this water sample is consistent with the results obtained for the Copano Bay oyster samples, supporting previous evidence that agricultural runoff enters Copano Bay (197).

Members of the subclass *Synechococcophycideae* were the most abundant in all of the Galveston Bay and Nueces Bay oyster samples. This subclass is part of the cyanobacterial class *Cyanophyceae*. Cyanobacteria are largely ubiquitous and very diverse, with members of the phylum able to utilize nitrate, heavy metal ions, and ammonium as nutrients (201). Recent studies have proposed using cyanobacteria as bioremediators of water affected by urban and industrial pollution, since they readily consume toxic compounds (such as mercury compounds and phenol) found in industrial effluents and wastewater (201-204). Since dominance of *Synechococcophycideae* is indicative of urban or industrial waste pollution, the dominance of

this subclass in Galveston Bay and Nueces Bay strongly suggests these bays receive these contaminants. While cyanobacteria have been found to dominate eastern oyster gut, tissue, and mantle microbiomes (> 70%), they have not been described as oyster symbionts or as part of their true core microbiome (205, 206).

Gammaproteobacteria was the dominant bacterial class in the Galveston Bay water sample and one 1852 Pass oyster sample. This class is large, diverse, and holds many pathogenic genera, such as *Vibrio*, *Salmonella*, and *Yersinia* (207). The dominance of the *Gammaproteobacteria* in place of other classes in coastal marine environments has been linked to elevated nutrient levels and contamination by hydrocarbons, heavy metals, and chemicals (208). Additionally, *Gammaproteobacteria* have been found to constitute the greatest percentage of ARB in water and sediment samples collected from aquaculture farms (208, 209). High numbers of *Gammaproteobacteria* in coastal waters near human population centers may be indicative of sewage pollution, especially if most of the detected bacteria belong to the family *Enterobacteriaceae* (210).

Mollicutes was the most abundant class in two of the 1852 Pass oyster samples, constituting 41.2 and 66.5% of each community. This is a unique class of bacteria, with its members known to contain the smallest genomes of any living cells (211). These bacteria can have symbiotic relationships with oysters and many other types of animals, acting as pathogens or commensal flora (206, 212-215). Members of the class *Mollicutes* (the genus *Mycoplasma* in particular) have been previously found in oyster gastrointestinal tracts in large numbers (206). These ecological roles likely lend to the high abundance of sequences belonging to this taxon in the oyster sample pools and the relative lack of them in the water samples.

The *Alphaproteobacteria* were the most abundant of all taxa in the Nueces Bay and 1852 Pass water samples. These bacteria have diverse morphologies and reproductive strategies; some exhibit stalks and/or undergo budding (216). This class contains the SAR11 clade, which is the most abundant taxonomic group of bacteria in the marine environment (217). The microbial communities in these two samples resemble those isolated from other coastal marine environments around the world (208, 218). The high percentage of sequences belonging to this taxon in the Nueces Bay water sample is likely due to the association of Alphaproteobacteria with mangroves and other plant life, as this site was adjacent to a large stand of black mangroves (*Avicennia germinans*) and saltmarsh cordgrass (*Spartina alterniflora*) (219-221). The dominance of this class in the 1852 Pass water sample could reflect the site's frequent exchange of water with the Gulf of Mexico.

Principal coordinate analysis of taxonomy revealed an unexpectedly high degree of similarity between all water sample communities, shedding light on the similarity of water microbiota in discrete locations and the effect of local wastewater inflows on different types of microbial communities. The minimum distance between any two sampling sites was 40 km (252 km maximum), but all of the water sample communities clustered together. It is possible that seawater bacterial communities may be relatively similar throughout the region of the Texas coast. The water sample communities clustered together and the oyster sample communities differed between (but not within) sites. When the effect of major wastewater inflow type on community composition is also considered, it seems that local conditions (inflows) select for distinct oyster microbiota, but not for distinct free-living microbiota in the overlying water. Therefore, when attempting to observe the effects of different types of wastewater inflows on microbial communities, greatest resolution may be achieved if oysters are examined. Since these

inflows carry ARB and ARGs, it follows that oysters serve as promising sentinel organisms for the detection of these elements.

It is difficult to determine why the abundance, richness, relative diversity, and evenness of the ARGs varied so widely between samples and sites; there are many factors that may have contributed to these quantities. The abundance and richness of ARGs were not found to have a statistical relationship with any environmental parameter or biological metric (oyster mass, DNA yield, etc.). Without additional data detailing the hydrochemistry, nutrient levels, and pollutant composition of the water at each site or long-term environmental data, it is unclear why there were as many ARGs and ARG types as there were. Based on the established body of knowledge, residual antibiotics from wastewater, allelopathic compounds, excess nutrients, heavy metals, other toxic compounds, and the influx of wastewater-associated bacteria likely contributed to these numbers (12, 16, 24, 56, 139, 140, 147, 148, 150).

The most abundant ARGs found across all samples were the mutated *tuf*, *rpoB*, and *gyrB* genes. The mutated *tuf* gene was the most abundant ARG in half of the samples, specifically the Galveston Bay oyster samples, the Nueces Bay oyster sample, and the 1852 Pass water sample. The mutated gene confers resistance against elfamycins (protein synthesis inhibitors), such as kirromycin, which target elongation factor Tu (222, 223). More importantly, this gene, mutant and otherwise, is widely conserved among bacteria and has been used for identification purposes, similar to 16S rRNA gene sequence analysis (224-226). It has even been described as more discriminatory than 16S rRNA-based analysis, specifically for identifying clinically-derived coagulase-negative Staphylococci (226). The abundance of this mutated gene in the samples is likely due to the gene's universal presence in bacteria (225).

The mutated *rpoB* gene was the most abundant ARG in the Copano Bay oyster and water samples, the Galveston Bay water sample, and the Nueces Bay water sample. Mutations in this gene confer resistance against rifamycins, such as rifampicin, which are mainly administered for the treatment of mycobacterial infections (3, 113). Similar to the 16S rRNA gene and *tuf*, this gene is widely conserved among bacteria and has been described as an effective tool for the identification of bacteria (227). Its abundance in the samples is also likely due to its ubiquity in bacteria.

The mutated *gyrB* gene was the most abundant ARG in the 1852 Pass oyster samples. Mutations in this gene confer resistance against quinolones, such as ciprofloxacin, which target bacterial DNA gyrase (64). Similar to the 16S rRNA gene, *tuf*, and *rpoB*, this gene is largely universal in bacteria, it has been used for taxonomic identification purposes, and its abundance is likely due to its ubiquity in bacteria (228).

The *blaTEM* gene, which was found solely in the 1852 Pass oyster samples, is of particular interest since its presence indicates anthropogenic antibiotic resistance contamination. Two *blaTEM* genes were found in the 1852 Pass oyster samples. This gene encodes the Class A β-lactamase TEM, the most common β-lactamase (229). It can hydrolyze penicillins, cephalosporins, and monobactams and has been found in *E. coli*, *K. pneumoniae*, and *Neisseria gonorrhoeae* (229-231). It was recently demonstrated that the *blaTEM* gene may serve as an effective indicator of antibiotic resistance contamination in WWTP-treated and -untreated hospital effluent (232). It follows that this gene could also be used as an indicator of resistance in coastal environments, which are nearly always downstream of WWTPs.

CHAPTER VI: SUMMARY AND CONCLUSIONS

Through shotgun metagenomic DNA sequencing and the use of bioinformatics tools, the microbial communities of oyster and water samples from Copano Bay, Galveston Bay, Nueces Bay, and 1852 Pass were characterized and ARG abundance and richness in each sample was determined. This study contributed to the development of methods to investigate the abundance and richness of ARGs in the coastal environment and produced baseline data detailing the abundance and richness of ARGs in four sites along the Texas coast.

Analysis of the microbial community of each sample provided insight into each site's hydrochemistry. A possible relationship between the dominant bacterial class in each of the samples and each site's major type of wastewater input was elucidated. This helps to confirm the prediction that Copano Bay, Galveston Bay, and Nueces Bay mainly receive agricultural runoff, urban wastewater, and industrial pollution, respectively. The microbial communities did not cluster as expected; microbial assemblages of the water samples were distinct in comparison to the oyster samples, suggesting that these free-living communities are similar throughout the study region.

The ARGs that were in the samples were identified. This allows future research efforts to monitor their development in these sites and their spread into others. Contrary to this study's predictions, ARG abundance and richness in the oyster samples was significantly lower, on average, than in the water samples. These findings suggest that oysters may be buffered from the bioaccumulation of ARGs. Alternatively, the abundance of bacterial DNA sequence reads may have been diminished by the presence of eukaryotic DNA, resulting in fewer detected ARGs.

The potential of the eastern oyster (*C. virginica*) to serve as an indicator species for the detection of ARGs was also evaluated. Metagenomic data from the oyster samples revealed the

presence of ARGs that were not detected in the water samples. The primary example of this is the presence of the *blaTEM* gene in the 1852 Pass oyster sample. As this gene has been described as an indicator of antibiotic resistance contamination from hospital effluent, its detection was highly valuable (232). As evidenced by the findings of this study, the eastern oyster proved to be a promising indicator species for the detection of ARGs in the coastal environment.

Future directions for research include testing water chemistry to determine nutrient levels and pollutant composition in each water body to investigate possible relationships between ARG abundance and richness with pollutants. Additionally, future research efforts should be directed at evaluating ARG abundance and richness in sediment samples from these sites.

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APPENDIX

Supplementary material.

Table S1. Measurements of oysters collected from Copano Bay, Galveston Bay, Nueces Bay, and 1852 Pass, Texas. Oysters were pooled (3 pools of 4 oysters per site).

Site	Sample	Oyster	Length (mm)	Wet Body Mass (g)	Wet Stomach Pool Mass (g)
Galveston Bay	GO1	1	102	19.435	
		2	83	12.321	
		3	88	13.686	
		4	76	10.83	0.768
	GO2	5	77	11.05	
		6	80	8.792	
		7	83	9.778	
		8	102	20.863	0.748
	GO3	9	87	9.162	
		10	84	14.128	
		11	87	10.476	
		12	82	13.646	0.761
Copano Bay	CO1	1	77	8.142	
		2	76	5.092	
		3	95	8.184	
		4	82	11.181	0.762
	CO2	5	91	9.882	
		6	84	8.934	
		7	88	8.848	
		8	89	7.297	0.988
	CO3	9	87	9.404	
		10	85	12.736	
		11	82	4.821	
		12	80	9.921	1.018
Nueces Bay	NO1	1	91	13.558	
		2	105	12.072	
		3	116	22.186	
		4	107	26.675	0.79

	NO2	5	104	30.612	
		6	108	12.344	
		7	87	7.483	
		8	116	19.889	0.707
	NO3	9	113	19.552	
		10	133	23.236	
		11	79	7.532	
		12	101	15.977	0.788
1852 Pass	PO1	1	114	19.519	
		2	85	11.608	
		3	86	11.668	
		4	88	10.526	0.605
	PO2	5	90	19.172	
		6	80	9.347	
		7	82	11.134	
		8	93	11.555	0.705
	PO3	9	93	11.137	
		10	94	13.565	
		11	81	8.73	
		12	93	13.101	0.565

Table S2. DNA concentrations and purities of oyster and water samples collected from Galveston Bay, Copano Bay, Nueces Bay, and 1852 Pass, Texas. Total quantities of DNA were in 200µL of sterile deionized water. A BioPhotometer D30 (Eppendorf AG, Hamburg, Germany) was used for quantitation. The European Bioinformatics Institute Metagenomics analysis pipeline was used for read merging, trimming, and quality/adapter filtering.

Sample	DNA Concentration (ng/µL)	A260/ 1mm	A260/A 280	Number of Raw Reads (Paired-end)	Number of Reads (Merged)	Number of Reads (Post Trimming and Filtering)
GO1	27.4	0.055	1.7	18,793,022	27,832,976	25,955,494
GO2	10.5	0.021	1.49	17,395,265	27,823,959	25,814,154
GO3	59.6	0.124	1.78	15,373,166	21,417,299	19,579,712
GW1	23.4	0.058	1.55	18,378,227	24,681,231	22,567,096
GW2	24.5	0.056	1.54			
GW3	31.7	0.081	1.82			
CO1	27.7	0.055	1.72	20,137,472	35,402,484	33,317,124
CO2	9.6	0.019	1.79	23,964,537	40,468,458	38,065,049
CO3	5.8	0.011	2.33	25,627,760	34,685,017	31,071,164
CW1	34.7	0.106	1.78	18,872,195	35,604,587	33,645,422
CW2	35.9	0.126	1.76			
CW3	336	0.108	1.79			
NO1	14.9	0.03	1.87	24,010,589	43,908,181	41,070,825
NO2	12.9	0.026	1.88	23,582,737	43,064,449	40,238,438
NO3	9.2	0.018	2.02	26,516,228	46,921,230	43,707,231
NW1	40.4	0.118	1.83	30,674,686	50,324,190	48,330,505
NW2	47.1	0.126	1.84			
NW3	50.9	0.131	1.85			
PO1	8.9	0.019	1.86	17,174,297	20,664,758	15,227,437
PO2	9.1	0.019	1.74	24,765,600	40,516,930	38,143,668
PO3	14.2	0.029	1.72	27,271,089	44,424,788	42,044,347
PW1	13.2	0.026	1.93	22,031,853	38,826,560	36,851,021
PW2	14.1	0.028	1.89			
PW3	12	0.024	1.84			

Table S3. DNA concentrations of oyster and water samples collected from Copano Bay, Galveston Bay, Nueces Bay, and 1852 Pass, Texas. Total quantities of DNA were in 200μL of sterile deionized water. A Qubit® Fluorometer (ThermoFisher Scientific, Lenexa, TX) was used for quantitation.

Sample	DNA Concentration (ng/mL)
GO1	4.66E+03
GO2	1.28E+03
GO3	1.51E+04
GW1	4.02E+03
GW2	4.18E+03
GW3	2.18E+04
CO1	3.46E+03
CO2	2.66E+03
CO3	344
CW1	1.57E+04
CW2	2.14E+04
CW3	2.00E+04
NO1	9.70E+03
NO2	6.16E+03
NO3	3.80E+03
NW1	2.54E+04
NW2	2.70E+04
NW3	3.22E+04
PO1	2.76E+03
PO2	1.78E+03
PO3	1.66E+03
PW1	1.09E+04
PW2	1.10E+04
PW3	8.52E+03

Table S4. Average DNA library size of each of the oyster and water samples collected from Galveston Bay, Copano Bay, Nueces Bay, and 1852 Pass, Texas prior to DNA sequencing. Water samples were pooled for each site.

Sample	Average Library Size (bp)
GO1	733
GO2	1040
GO3	470
GW1	620
GW2	Combined with GW1
GW3	Combined with GW1
CO1	1225
CO2	976
CO3	1459
CW1	1391
CW2	Combined with CW1
CW3	Combined with CW1
PO1	499
PO2	1014
PO3	1002
PW1	829
PW2	Combined with PW1
PW3	Combined with PW1
NO1	1045
NO2	1148
NO3	1302
NW1	719
NW2	Combined with NW1
NW3	Combined with NW1

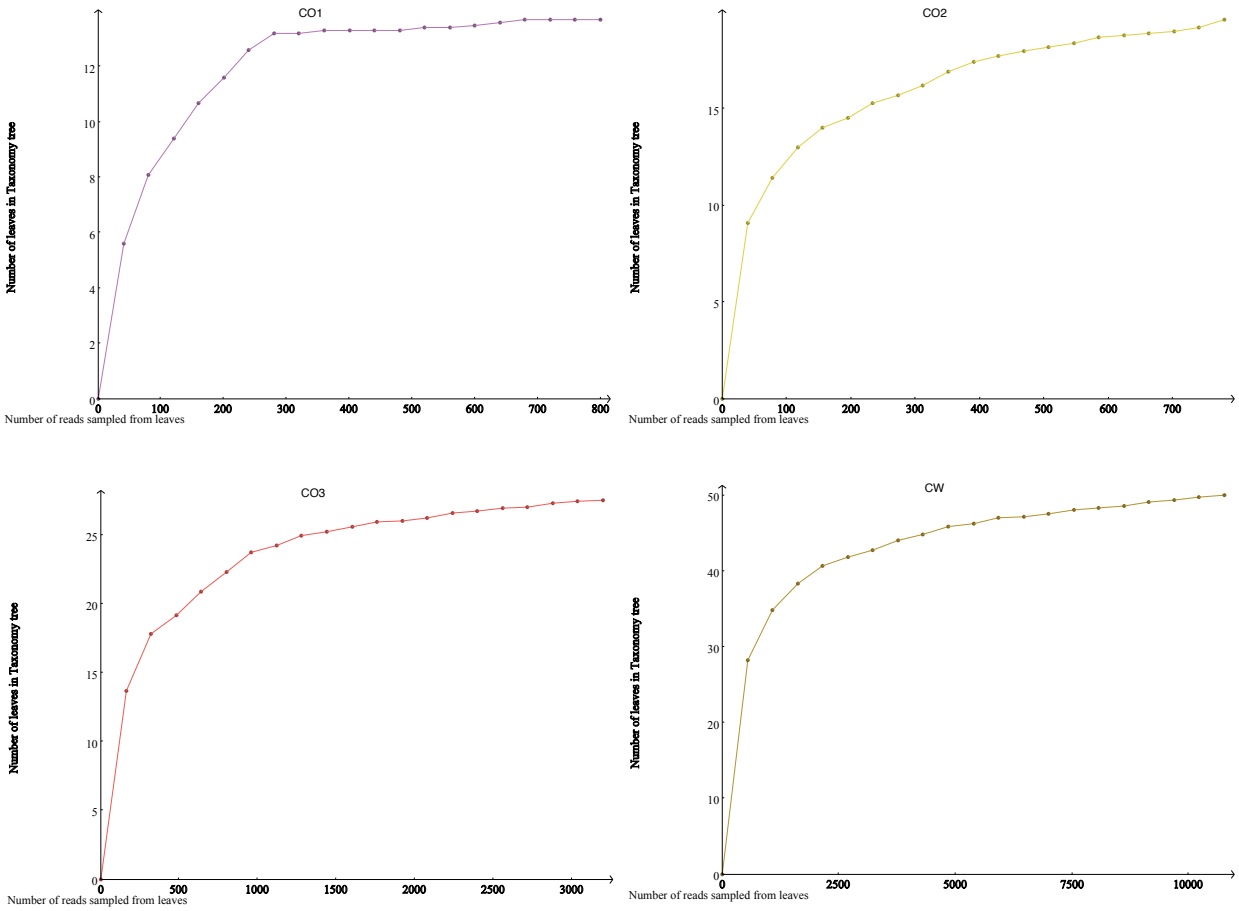

Figure S1. Rarefaction curves of each oyster and water sample metagenome from the Copano Bay sampling site. The rarefaction curves were created based on taxonomic class and number of reads.

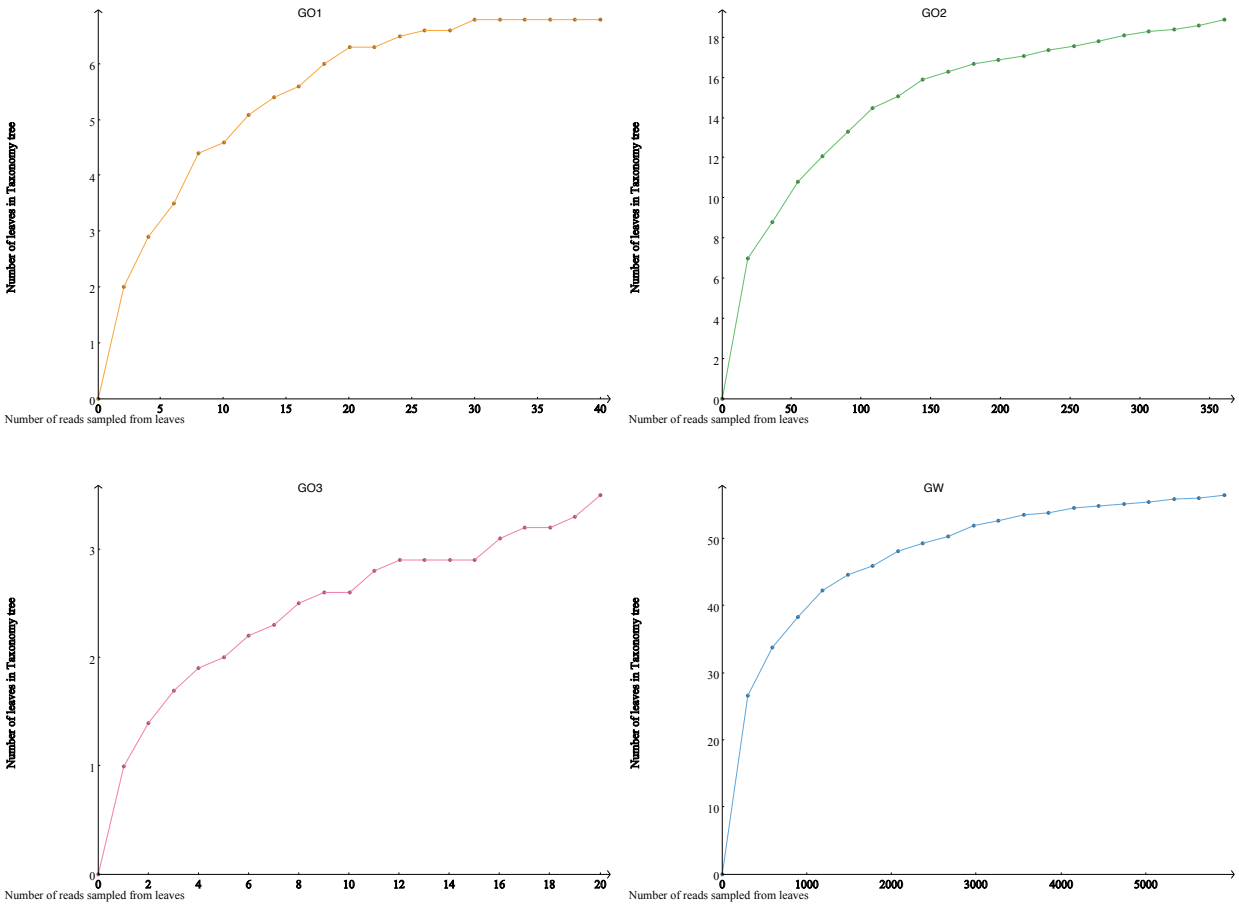

Figure S2. Rarefaction curves of each oyster and water sample metagenome from the Galveston Bay sampling site. The rarefaction curves were created based on taxonomic class and number of reads.

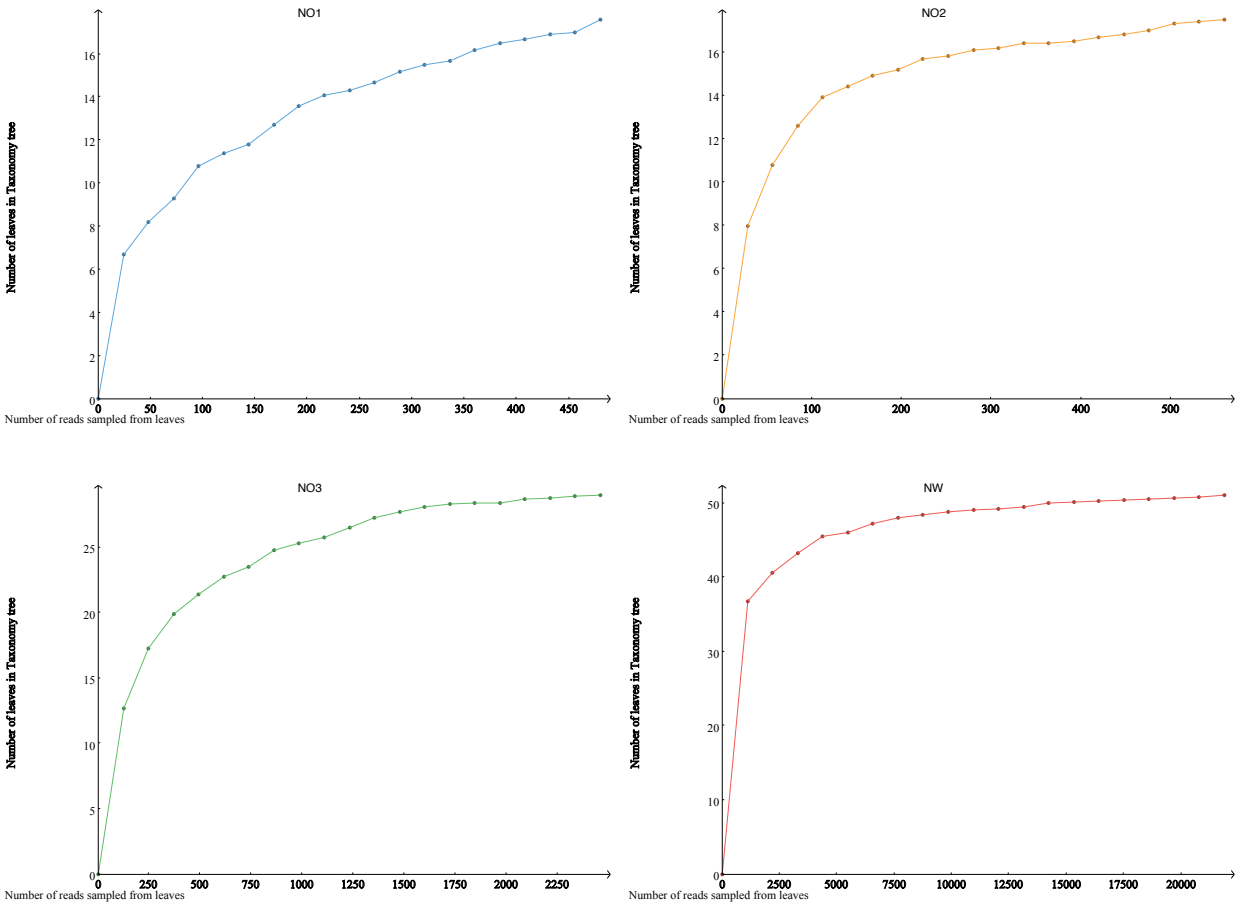

Figure S3. Rarefaction curves of each oyster and water sample metagenome from the Nueces Bay sampling site. The rarefaction curves were created based on taxonomic class and number of reads.

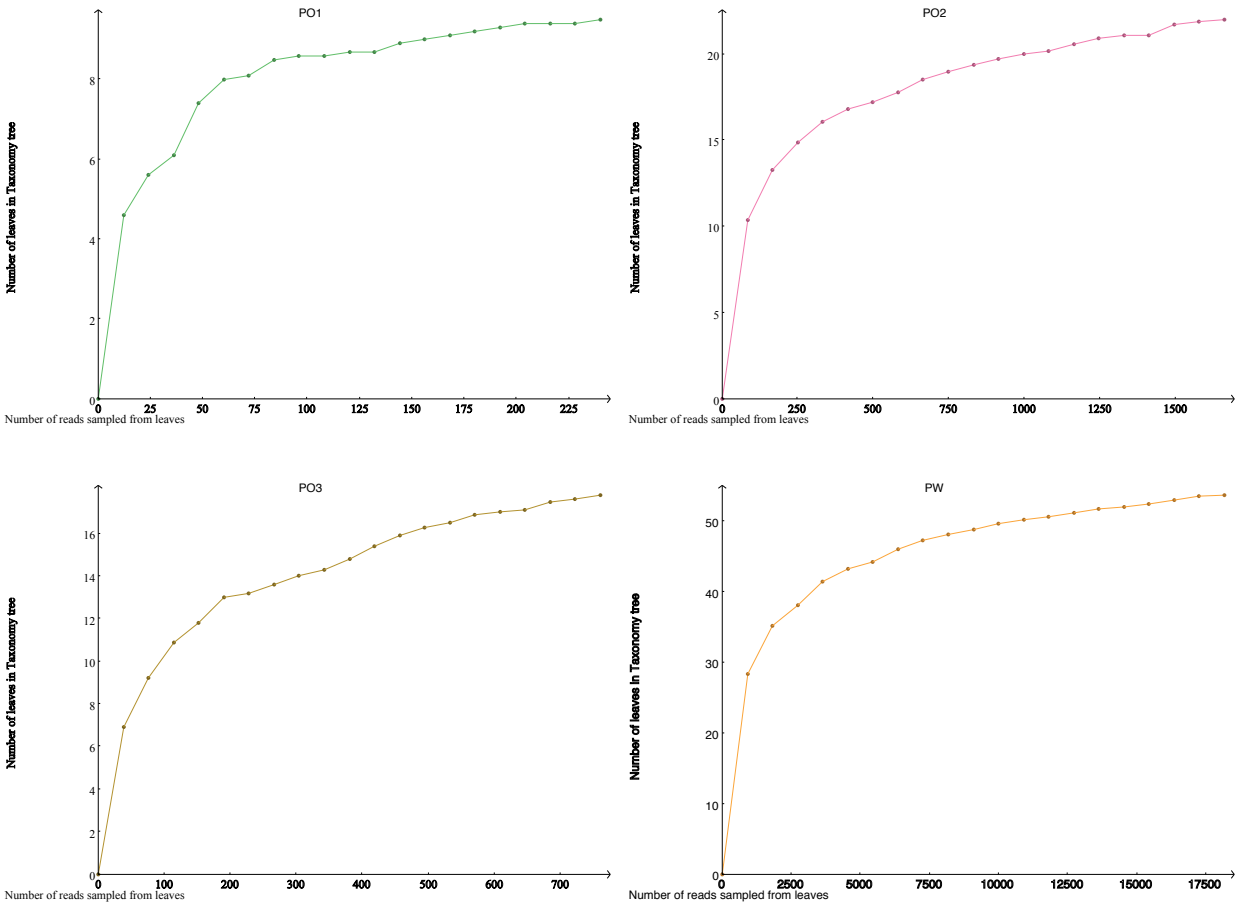

Figure S4. Rarefaction curves of each oyster and water sample metagenome from the 1852 Pass sampling site. The rarefaction curves were created based on taxonomic class and number of reads.

Table S5. Antibiotic resistance gene (ARG) identities and abundance in the oyster and water samples collected from Copano Bay, Galveston Bay, Nueces Bay, and 1852 Pass, Texas.

	CO	CW	GO	GW	NO	NW	PO	PW
acrB	0	2	0	1	0	0	0	0
acrD	0	0	0	2	0	0	0	1
amrB	0	1	0	0	0	0	0	1
ant	1	1	0	0	0	1	0	0
arnA	0	0	0	0	0	0	0	1
blaCTX	0	0	1	0	3	0	0	1
blaOXA	1	0	0	0	3	1	0	0
blaTEM	0	0	0	0	0	0	2	0
blaVEB	0	0	0	0	0	0	0	1
carA	0	1	0	0	0	0	0	1
carB	0	0	1	0	2	0	0	4
ceoB	0	1	2	1	0	3	0	2
drrA	0	0	0	0	0	4	0	0
embB	0	3	0	1	0	0	0	0
facT	0	2	0	0	0	0	0	0
folP	5	13	0	7	0	2	0	2
gyrA	23	106	4	75	13	143	9	104
gyrB	20	15	1	30	35	32	103	34
hlyD	0	0	0	1	0	0	0	0
kasA	0	0	0	0	0	1	0	0
macB	0	7	0	6	0	1	0	1
mdsB	0	0	0	0	0	1	0	1
mdtC	2	5	1	1	1	1	0	0
mexB	4	8	0	6	11	8	0	2
mexC	0	0	0	0	0	0	0	1
mexD	2	4	0	2	0	5	2	5
mexF	5	7	0	9	0	17	1	9
mexK	0	1	0	0	0	0	0	0
mexN	0	1	0	1	0	0	0	0
mexQ	0	2	0	1	1	4	1	2
mexW	0	6	0	3	0	4	1	0
msrA	0	0	0	0	0	1	0	0
mtrD	4	2	3	1	0	4	0	2
novA	0	1	0	0	0	0	0	0
oleB	0	1	0	0	0	2	0	0
ompR	0	0	0	1	0	2	0	0
oqxB	1	0	0	4	0	1	0	1
otrA	0	0	0	0	0	1	0	1
parC	0	8	0	4	14	7	0	5
parE	6	25	1	30	10	43	5	55
pmrA	0	42	0	13	12	25	6	7
rph	0	0	0	1	0	0	0	0
rpoB	49	388	7	185	58	293	37	213
rpoC	1	0	0	2	2	5	0	9
rpsL	2	7	0	3	0	1	0	2
SAV1866	0	0	0	0	0	0	0	1
sme	1	4	0	5	0	2	0	4
srmB	0	0	0	2	0	0	0	0
tet35	0	0	0	0	0	0	1	1
tetR	0	0	0	0	0	0	0	2
tlrC	0	5	0	2	4	3	0	0
tuf	31	157	7	133	60	193	62	224
vanRO	0	0	0	0	0	1	0	0
TOTAL	**158**	**826**	**28**	**533**	**229**	**812**	**230**	**700**