Characterizing Bacterial Antibiotic Resistance, Prevalence, and Persistence in the Marine Environment

By

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Submitted to Massachusetts Institute of Technology and the Woods Hole Oceanographic Institution on January 3, 2019, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Marine Environmental Microbiology.

ABSTRACT

Antibiotics are naturally occurring chemicals in bacteria that were recently discovered and utilized by humans. Despite a relatively short time of use, anthropogenic use of antibiotics has increased natural levels of antibiotic resistance, which has caused a looming antibiotic resistance crisis, where antibiotics may not work. Understanding resistance patterns is critical to allow for continued therapeutic use of antibiotics. While resistance is often thought of in hospitals, antibiotics and antibiotic resistance genes from human activity are disposed of into nature where they are able to interact with naturally occurring antibiotics and resistance. In this dissertation, I examine the ocean as an understudied region of the environment for antibiotic resistance. The ocean represents an area of human activity with recreation and food consumption and it is an enormous region of the planet that is affected by both land and sea activities. In Chapter 2, I explore the policies that have contributed to the antibiotic resistance crisis. I offer explanations of market and political failures that contributed to the situation, areas for growth in terms of assessing scientific knowledge, and finally, recommendations for mitigating antibiotic resistance. In Chapters 3 and 4, I collected individual bacterial cultures from Cape Cod, MA beaches to assess the phenotypic response to antibiotic resistance. I show that 73% of *Vibrio*-like bacteria and 95% of heterotrophic bacteria (both groups operationally defined) are resistant to at least one antibiotic. These results indicate that antibiotic resistance is prevalent and persistent on beaches over both spatial and temporal scales. In Chapter 5, I used metagenomics to assess the abundance and types of resistance genes at coastal impacted Massachusetts sites. I found that, even in sites that seem distinct in terms of anthropogenic impact, prevalence of resistance remained the same. Finally, in Appendix A, I examined part of the TARA Ocean dataset for prevalence of antibiotic resistance genes across the world's ocean. Here, I found that there are distinctions between different ocean biomes based upon antibiotic, metal, and mobile genetic elements. This dissertation has increased the understanding of temporal and spatial dynamics of antibiotic resistance in the coastal and open ocean.

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DEDICATION

This dissertation is dedicated to the scientist in all of us from age 0 to age 101+. May all continue to work for our systems and societies to make science more accessible to everyone.

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CHAPTER 1: Introduction

Introduction to Antibiotics and Antibiotic Resistance

An antibiotic is a "compound produced by a microorganism that inhibits the growth of another microorganism" (1). Antibiotics are natural products and have existed in nature for many years. The exact time of antibiotic production is unknown currently, but estimates include from 2 billion years ago to 30 million years ago (2). Baltz's estimates that aceinomycetes bacteria have produced antibiotics for a million years (3). Antibiotics were detected by humans in 1928 with Alexander Fleming's discovery of penicillin (4). However, there is evidence for human use of antibiotics from ancient times (5). From the discovery of penicillin to vancomycin and rifamycin, these molecules have revolutionized the way humans interact with the world. These antibiotics have a variety of cellular targets to inhibit growth of bacteria or to kill them (Figure 1) (6). These molecules were quickly utilized in a variety of ways ranging from treatment of human (7, 8) and animal health issues (9) to sub-therapeutic use in animals for food production (1, 7, 10, 11) and to treatment of plants against disease (1, 7, 10). Use of these drugs changed previously life threatening bacterial illnesses to easily cured ailments, leading antibiotics to be called "miracle drugs" (12). Despite their dramatic usefulness for the treatment of many diseases, the long-term effectiveness of these "miracle drugs" has recently been called into question (4, 8, 13–18). The widespread use of antibiotics throughout our society has contributed to increased levels of antibiotic resistance within microbes and within the environment.

Like antibiotics, antibiotic resistance (AR) is a naturally occurring phenomenon where an organism is invulnerable to a given chemical. AR has been found in many isolated environments such as remote caves (19), ancient terrestrial sediments (2), and in the microbiomes of humans isolated from "modern" societies (20). At its most basic level,

presence of antibiotics in an environment selects for antibiotic resistant bacteria (ARB) (8). In human use of antibiotics, the purpose is to damage or eradicate the bacteria that are causing the infection; but cells that are unaffected by the antibiotic are able to continue growing. In animal production, antibiotics are often fed to animals at subtherapeutic levels to enhance growth, allowing producers to bring the animals to market more quickly (21, 22), but also unintentionally selecting for the growth/persistence of resistant bacteria. Many bacteria can participate in horizontal gene transfer (HGT), facilitating sharing antibiotic resistance genes between bacteria of the same or different species (8, 14). While the existence of resistance is not dependent on humanity's use of antibiotics, the prevalence of their use contributes to an increase in antibiotic resistant bacteria (7).

Without better stewardship with respect to antibiotic applications, humans may enter into a Post-Antibiotic Era, a time where a majority of presently known antibiotics do not work to treat bacterial infections and many now commonplace surgeries would not be safe due to the threat of infection (4). Antibiotic resistance causes at least two million infections and 23,000 deaths a year in the United States, which is likely an underestimate due to the lack of available data (4). Recent projections for 2050 anticipate ten million deaths worldwide from resistant infections each year, resulting in a global cumulative cost of $100 trillion United States dollars by 2050 (23).

Environmental Reservoirs of Resistance

With the substantial cost of AR anticipated to increase in the coming years, the Centers for Disease Control (4), World Health Organization (24), the United Nations (25) and the United States government (26) have all become more interested in creating strategies to

combat antibiotic resistance. In this effort, it is important to consider the effect of environmental reservoirs of resistance. Understanding the reservoirs is important because environments in which humans live, work, and play may serve as the source of resistance that presents in clinics (27). Interactions between humans do not stay in the clinic; and antibiotic resistant bacteria do not adhere to human boundaries. Pollution from anthropogenic sources such as hospitals (28–30), agriculture(13, 31–35), human wastewater (36–41), and aquaculture (42–46) can contain antibiotics along with bacteria that have antibiotic resistance genes (ARG). Up to 90% of the antibiotic dose passes through the body unchanged(47), showing how easily these chemicals can enter into waste products that are often routed into streams, rivers, or sediments in landfills. Once these wastes are released, they enter into the environment, adding to the amounts of antibiotics and ARG that occur naturally.

Although the presence of antibiotics can select for ARG in the environment (10, 17, 48–50), it is not required to stimulate the transfer of those genes from non-native bacteria to native environmental bacteria and vice versa (51). One of the driving forces behind the increase in environmental antibiotic resistance is the ease with which resistance genes can be transferred, even between distantly related taxa (52). As a consequence, environmental microbes that have little to no effect on human disease, or exposure to clinically relevant antibiotics, may acquire clinically relevant antibiotic resistant genes. Together, this creates an environment where resistance can be transferred and maintained within the bacterial community. As humans interact with the environment, they have the opportunity to acquire both pathogenic and non-pathogenic bacteria carrying resistance genes directly

by consumption of water, animals, and plants, or indirectly through recreational activity such as swimming. Therefore, the environment represents a reservoir for resistance, and the potential for resulting in resistant infections (17, 48, 49, 53).

While the knowledge that the environment is important in antibiotic resistance has been disseminated in a wide variety of review and policy papers (1, 10, 11, 17, 48, 49, 53–63), original research on resistance in the environment pales in comparison to clinical research. The majority of environmental research is not equally represented across field sites. Environmental research has focused on wastewater treatment effluents to the environment or on agriculture effluents to rivers and streams. The marine environment is one of the ultimate sites for anthropogenic pollution because the ocean is used as a dumping ground for wastes, either direct intentional releases or indirectly through polluted streams and rivers (Figure 2).

Marine Environment as a Resistance Reservoir

Early studies of marine AR found that it was present and suggested that more impacted areas had greater prevalence of resistance (64). Most studies have been descriptive regarding the resistance patterns within the marine environment: examining levels of resistance in pigmented versus non-pigmented bacteria (65), between bacteria in surface and subsurface water (66, 67), and within bacteria present in sand transects on the beach (68, 69). The hypothesis of the marine environment as a resistance reservoir has been strengthened by research revealing AR in bacteria present in marine animals (70–74) and in a variety of marine bacteria including heterotrophs (69, 75), fecal indicators (76–78), and potential pathogens (79–81).

Although recent studies have provided evidence of increased AR in areas with greater anthropogenic inputs (76, 82), resistance is also seen in the open ocean (83) and at isolated beaches (84). The majority of the world's populations lives within 400 km of a coast (4 billion people in 1998) (85). Humans routinely interact with ocean water during recreation, such as swimming or surfing, and through consumption of shellfish or fish. Fish consumption is not a small factor, as the ocean serves as the source of 17% of the world's animal protein (86). For humans, the concern is that resistance from environmental bacteria may be passed to a human's natural bacterial flora or to pathogenic bacteria during ingestion of raw shellfish or fish or through aquatic recreation. For example, the origin of a type of quinolone resistance (gene: gnrA), which has been problematic in the clinic, has been found to originate from *Shewanella*, a gamma Proteobacterium readily found in freshwater and marine environments (87).

The most recent research has attempted to assess the potential risks that AR in the ocean poses to humans. Leonard et al. 2015 examined the incidences of marine recreation activities that led to ingestion of resistant bacteria in England and Wales (88). They estimated that there were at least 6.3 million occurrences of ingestion in 2012 for *E.coli* containing resistance to the third-generation cephalosporins; and suggested that this is likely an underestimate of the risk of resistance acquisition from oceanic activity because they only examined one bacterial species and one antibiotic class for resistance (88).

Introduction to this Thesis

The lack of consistency between studies and the dearth of temporal-spatial studies on resistance in the marine environment makes it difficult to adequately and appropriately assess these potential human health risks and make appropriate societal recommendations. My doctoral research examines the levels of resistance in local coastal marine environments with relatively normal levels of human impact, surveys the prevalence and persistence of both resistant bacteria and ARG, and seeks to uncover what pollution inputs might be contributing to elevated levels of antibiotic resistance, in order to eventually inform assessment of human health risks.

To better understand resistance, Chapter 2 examines antibiotic resistance as a global problem from a policy standpoint and analyzes how this problem developed with market and political failures. The chapter explores what knowledge gaps remain in the clinical and environmental fields and provides recommendations that might be made to better preserve antibiotics. This chapter provides an understanding of the complicated nature of antibiotic resistance both in the political and scientific realms. Chapters 3-6 then examine environmental antibiotic resistance, which is shown to be understudied in Chapter 2. Chapters 3-6 provide greater knowledge of patterns of resistance in the environment.

The first two data chapters assess patterns of resistance in Cape Cod, MA at six different beaches over one year. This approach allows for assessing resistance over spatial distances with multiple sites, over temporal changes over one year, and finally over levels of human impact. It also allows estimation of the amount of antibiotic resistant bacteria encountered by humans through normal marine recreation or food consumption. Chapter 3 examines antibiotic resistance in *Vibrio*-like bacteria found on these beaches, while

Chapter 4 examines resistance in general heterotrophic bacteria. These chapters utilize cultivable bacteria and test them for their resistance to particular antibiotics, which represents antibiotic resistance that is being actively expressed phenotypically. For Chapter 3, five antibiotics were tested: amoxicillin (antibiotic mechanism - cell wall synthesis), ciprofloxacin (mechanism - DNA gyrase), doxycycline (mechanism - protein synthesis- 30S ribosomal subunit), oxytetracycline (mechanism - protein synthesis- 30S ribosomal subunit), and trimethoprim (mechanism - folic acid metabolism) (6). For Chapter 4, four antibiotics were tested: amoxicillin, ciprofloxacin, doxycycline, and erythromycin (mechanism: protein synthesis – 50S ribosomal subunit) (6).

However, the exact mechanism of resistance is not elucidated through this method. To examine resistance mechanisms, as indicated by antibiotic resistance genes present in the marine environment, metagenomic sequencing is employed. Metagenomic sequencing involves sequencing the total DNA present in an environmental sample, and can be used to examine a sample for a variety of resistance genes as well as their genomic context (location in plasmid or transposable element). This method is powerful because it allows analysis of many genes/antibiotics, which would not be cost or time effective when testing for resistance in the laboratory. Further, computational processing of metagenomic data can be used to discover new types of resistance genes. The drawback of this method is that these genes are simply present in the environment and we have no knowledge of if they are being actively expressed in a bacterium. In this light, metagenomic analyses should be seen as illustrating only the potential resistance of an environment instead of what is actively being expressed within living bacteria.

Chapter 5 and Appendix A include two metagenomic studies examining antibiotic resistance in the ocean. For Chapter 5, local coastal water and sediment samples were collected at industrial and wastewater impacted sites to examine if resistance genes vary based on human activity compared to reference sites. Appendix A provides a greater understanding of resistance in the global ocean by analyzing the open-access TARA Oceans data for antibiotic resistance prevalence and diversity. Overall, this dissertation provides an in depth look at antibiotic resistance present in the marine environment, using two methods—culture based resistance testing, illustrating active, phenotypic resistance, and metagenomic sequencing, indicating potential resistance.

References

1. **Kümmerer K**. 2009. Antibiotics in the aquatic environment--a review--part II. Chemosphere **75**:417–34.

2. **D'Costa VM**, **King CE**, **Kalan L**, **Morar M**, **Sung WWL**, **Schwarz C**, **Froese D**, **Zazula G**, **Calmels F**, **Debruyne R**, **Golding GB**, **Poinar HN**, **Wright GD**. 2011. Antibiotic resistance is ancient. Nature **477**:457–61.

3. **Baltz RH**. 2008. Renaissance in antibacterial discovery from actinomycetes. Curr. Opin. Pharmacol. **8**:557–563.

4. **Center for Disease Control and Prevention**. 2013. Antibiotic resistance threats in the United States, 2013.

5. **Aminov RI**. 2010. A brief history of the antibiotic era: Lessons learned and challenges for the future. Front. Microbiol. **1**:1–7.

6. **Lewis K**. 2013. Platforms for antibiotic discovery. Nat. Rev. Drug Discov. **12**:371–87.

7. **Martinez JL**. 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environ. Pollut. **157**:2893–2902.

8. **Alanis AJ**. Resistance to antibiotics: are we in the post-antibiotic era? Arch. Med. Res. **36**:697–705.

9. **Sarmah AK**, **Meyer MT**, **Boxall ABA**. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere **65**:725–59.

10. **Allen HK**, **Donato J**, **Wang HH**, **Cloud-Hansen K a**, **Davies J**, **Handelsman J**. 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. **8**:251–9.

11. **Kümmerer K**. 2004. Resistance in the environment. J. Antimicrob. Chemother. **54**:311–20.

12. **Sengupta S**, **Chattopadhyay MK**. 2012. Antibiotic Resistance of Bacteria : A Global Challenge 177–192.

13. **Silbergeld EK**, **Graham J**, **Price LB**. 2008. Industrial food animal production, antimicrobial resistance, and human health. Annu. Rev. Public Health **29**:151–69.

14. **Davies J**. 2006. Where have All the Antibiotics Gone? Can. J. Infect. Dis. Med. Microbiol. **17**:287–90.

15. **Wright GD**. 2013. Q&A: antibiotic resistance: what more do we know and what more can we do? BMC Biol. **11**:51.

16. **Spellberg B**, **Guidos R**, **Gilbert D**, **Bradley J**, **Boucher HW**, **Scheld WM**, **Bartlett JG**, **Edwards Jr. J**, **Edwards J**. 2008. The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. Clin. Infect. Dis. **46**:155–64.

17. **Ashbolt NJ**, **Amézquita A**, **Backhaus T**, **Borriello P**, **Brandt KK**. 2013. Human Health Risk Assessment (HHRA) for Environmental Development and Transfer of Antibiotic Resistance. Environ. Health Perspect. **121**:993–1002.

18. **European Centre for Disease Prevention and Control**, **European Medicines Agency**. 2009. The bacterial challenge : time to react.

19. **Bhullar K**, **Waglechner N**, **Pawlowski A**, **Koteva K**, **Banks ED**, **Johnston MD**, **Barton H a**, **Wright GD**. 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS One **7**:e34953.

20. **Clemente JC**, **Pehrsson EC**, **Blaser MJ**, **Sandhu K**, **Gao Z**, **Wang B**, **Magris M**, **Hidalgo G**, **Contreras M**, **Noya-Alarcon O**, **Lander O**, **McDonald J**, **Cox M**, **Walter J**, **Oh PL**, **Ruiz JF**, **Rodriguez S**, **Shen N**, **Song SJ**, **Metcalf J**, **Knight R**, **Dantas G**, **Dominguez-Bello MG**. 2015. The microbiome of uncontacted Amerindians. Sci. Adv. **1**:e1500183–e1500183.

21. **Moore PR**, **Evenson A**, **Luckey TD**, **McCoy E**, **Elvehjem CA**, **Hart EB**. 1946. Use of Sulfasuxidine, Streptothricin, and Streptomycin in Nutritional Studies with the Chick. J. Biol Chem. **165**:437–441.

22. **Jukes T.**, **Stoksad EL.**, **Taylor RR**, **Combs TJ**, **Edwards HM**, **Meadows GB**. 1950. Growth Promoting Effect of Aureomycin on Pigs. Arch. Biochem. **26**:324–330.

23. **Review on Antimicrobial Resistance**. 2014. Antimicrobial Resistance : Tackling a crisis for the health and wealth of nations.

24. **World Health Organization**. 2014. Antimicrobial Resistance: Global Report on surveillance.

25. **UN Environment (UNEP)**. 2017. Frontiers 2017: Emerging Issues of Environmental Concern, 1st ed. UNEP, Narobi.

26. **The White House**. 2015. National Action Plan for Combating Antibiotic-Resistant Bacteria. Washington D.C.

27. **Wright GD**. 2010. Antibiotic resistance in the environment: a link to the clinic? Curr. Opin. Microbiol. **13**:589–94.

28. **Jakobsen L**, **Sandvang D**, **Hansen LH**, **Bagger-Skjøt L**, **Westh H**, **Jørgensen C**, **Hansen DS**, **Pedersen BM**, **Monnet DL**, **Frimodt-Møller N**, **Sørensen SJ**, **Hammerum AM**. 2008. Characterisation, dissemination and persistence of gentamicin resistant *Escherichia coli* from a Danish university hospital to the waste water environment. Environ. Int. **34**:108–15.

29. **Chang X**, **Meyer MT**, **Liu X**, **Zhao Q**, **Chen H**, **Chen J**, **Qiu Z**, **Yang L**, **Cao J**, **Shu W**. 2010. Determination of antibiotics in sewage from hospitals, nursery and slaughter house, wastewater treatment plant and source water in Chongqing region of Three Gorge Reservoir in China. Environ. Pollut. **158**:1444–50.

30. **Thompson JM**, **Gündoğdu A**, **Stratton HM**, **Katouli M**. 2013. Antibiotic resistant *Staphylococcus aureus* in hospital wastewaters and sewage treatment plants with special reference to methicillin-resistant *Staphylococcus aureus* (MRSA). J. Appl. Microbiol.

114:44–54.

31. **Li L**, **Sun J**, **Liu B**, **Zhao D**, **Ma J**, **Deng H**, **Li X**, **Hu F**, **Liao X**, **Liu Y**. 2013. Quantification of lincomycin resistance genes associated with lincomycin residues in waters and soils adjacent to representative swine farms in China. Front. Microbiol. **4**:1–9.

32. **Chee-Sanford JC**, **Mackie RI**, **Koike S**, **Krapac IG**, **Lin Y-F**, **Yannarell AC**, **Maxwell S**, **Aminov RI**. 2009. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. J. Environ. Qual. **38**:1086–108.

33. **Ghosh S**, **LaPara TM**. 2007. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J. **1**:191–203.

34. **Sapkota AR**, **Hulet RM**, **Zhang G**, **McDermott P**, **Kinney EL**, **Schwab KJ**. 2011. Lower prevalence of antibiotic-resistance Enterococci on U.S. conventional poultry farms that transitioned to organic practices. Environ. Health Perspect. **119**:1622–1629.

35. **McEachran AD**, **Blackwell BR**, **Hanson JD**, **Wooten KJ**, **Mayer GD**, **Cox SB**, **Smith PN**. 2015. Antibiotics, Bacteria, and Antibiotic Resistance Genes: Aerial Transport from Cattle Feed Yards via Particulate Matter. Environ. Health Perspect. **123**:337–343.

36. **Bessa L**, **Barbosa-Vasconcelos A**, **Mendes Â**, **Vaz-Pires P**, **Martins da Costa P**. 2014. High prevalence of multidrug-resistant *Escherichia coli* and *Enterococcus spp.* in river water, upstream and downstream of a wastewater treatment plant. J. Water Health 426–436.

37. **Amos GCA**, **Zhang L**, **Hawkey PM**, **Gaze WH**, **Wellington EM**. 2014. Functional metagenomic analysis reveals rivers are a reservoir for diverse antibiotic resistance genes. Vet. Microbiol. **171**:441–447.

38. **Marti E**, **Jofre J**, **Balcazar JL**. 2013. Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. PLoS One **8**:1–8.

39. **Novo A**, **André S**, **Viana P**, **Nunes OC**, **Manaia CM**. 2013. Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. Water Res. **47**:1875–87.

40. **Bouki C**, **Venieri D**, **Diamadopoulos E**. 2013. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: A review. Ecotoxicol. Environ. Saf. **91**:1–9.

41. **Rizzo L**, **Manaia C**, **Merlin C**, **Schwartz T**, **Dagot C**, **Ploy MC**, **Michael I**, **Fatta-Kassinos D**. 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. **447**:345–360.

42. **Labella A**, **Gennari M**, **Ghidini V**, **Trento I**, **Manfrin A**, **Borrego JJ**, **Lleo MM**. 2013. High incidence of antibiotic multi-resistant bacteria in coastal areas dedicated to fish farming. Mar. Pollut. Bull. **70**:197–203.

43. **Di Cesare A**, **Luna GM**, **Vignaroli C**, **Pasquaroli S**, **Tota S**, **Paroncini P**, **Biavasco F**. 2013. Aquaculture can promote the presence and spread of antibiotic-resistant *Enterococci* in

marine sediments. PLoS One **8**:1–8.

44. **Yang J**, **Wang C**, **Shu C**, **Liu L**, **Geng J**, **Hu S**, **Feng J**. 2013. Marine sediment bacteria harbor antibiotic resistance genes highly similar to those found in human pathogens. Microb. Ecol. **65**:975–981.

45. **Chelossi E**, **Vezzulli L**, **Milano A**, **Branzoni M**, **Fabiano M**, **Riccardi G**, **Banat IM**. 2003. Antibiotic resistance of benthic bacteria in fish-farm and control sediments of the Western Mediterranean. Aquaculture **219**:83–97.

46. **Buschmann AH**, **Tomova A**, **López A**, **Maldonado MA**, **Henríquez LA**, **Ivanova L**, **Moy F**, **Godfrey HP**, **Cabello FC**. 2012. Salmon aquaculture and antimicrobial resistance in the marine environment. PLoS One **7**:26–28.

47. **Kumar K**, **Gupta SC**, **Baidoo SK**, **Chander Y**, **Rosen CJ**. 2005. Antibiotic uptake by plants from soil fertilized with animal manure. J. Environ. Qual. **34**:2082–5.

48. **Cantas L**, **Shah SQ a**, **Cavaco LM**, **Manaia CM**, **Walsh F**, **Popowska M**, **Garelick H**, **Bürgmann H**, **Sørum H**. 2013. A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota. Front. Microbiol. **4**:1–14.

49. **Finley RL**, **Collignon P**, **Larsson DGJ**, **Mcewen SA**, **Li XZ**, **Gaze WH**, **Reid-Smith R**, **Timinouni M**, **Graham DW**, **Topp E**. 2013. The scourge of antibiotic resistance: The important role of the environment. Clin. Infect. Dis. **57**:704–710.

50. **Jiang SC**, **Paul JH**. 1998. Gene Transfer by Transduction in the Marine Environment Gene Transfer by Transduction in the Marine Environment **64**:2780–2787.

51. **Alm EW**, **Zimbler D**, **Callahan E**, **Plomaritis E**. 2014. Patterns and persistence of antibiotic resistance in faecal indicator bacteria from freshwater recreational beaches. J. Appl. Microbiol. **117**:273–85.

52. **Salyers AA**, **Amabile-Cuevas CF**. 1997. Why Are Antibiotic Resistance Genes So Resistant to Elimination ? Antimicrobal Agents Chemother. **41**:2321–2325.

53. **Pruden A**, **Larsson DGJ**, **Amézquita A**, **Collignon P**, **Brandt KK**, **Graham DW**, **Lazorchak JM**, **Suzuki S**, **Silley P**, **Snape JR**, **Topp E**, **Zhang T**, **Zhu Y-G**. 2013. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. Environ. Health Perspect. **121**:878–85.

54. **Berendonk TU**, **Manaia CM**, **Merlin C**, **Fatta-Kassinos D**, **Cytryn E**, **Walsh F**, **Bürgmann H**, **Sørum H**, **Norström M**, **Pons M-N**, **Kreuzinger N**, **Huovinen P**, **Stefani S**, **Schwartz T**, **Kisand V**, **Baquero F**, **Martinez JL**. 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 1–8.

55. **Cantón R**. 2009. Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting. Clin. Microbiol. Infect. **15 Suppl 1**:20–25.

56. **Baquero F**, **Martínez J-L**, **Cantón R**. 2008. Antibiotics and antibiotic resistance in water

environments. Curr. Opin. Biotechnol. **19**:260–5.

57. **da Costa PM**, **Loureiro LL**, **Matos AJF**. 2013. Transfer of multidrug-resistant bacteria between intermingled ecological niches: The interface between humans, animals and the environment. Int. J. Environ. Res. Public Health **10**:278–294.

58. **Gaze W**, ***O'*Neill C**, **Wellington E**, **Hawkey P**. 2008. Antibiotic resistance in the environment, with particular reference to MRSA. Adv. Appl. Microbiol. **63**:249–280.

59. **Kümmerer K**. 2009. Antibiotics in the aquatic environment--a review--part II. Chemosphere **75**:435–41.

60. **Marti E**, **Variatza E**, **Balcazar JL**. 2014. The role of aquatic ecosystems as reservoirs of antibiotic resistance. Trends Microbiol. **22**:36–41.

61. **Wellington EMH**, **Boxall ABA**, **Cross P**, **Feil EJ**, **Gaze WH**, **Hawkey PM**, **Johnson-Rollings AS**, **Jones DL**, **Lee NM**, **Otten W**, **Thomas CM**, **Williams AP**. 2013. The role of the natural environment in the emergence of antibiotic resistance in gram-negative bacteria. Lancet Infect. Dis. **13**:155–65.

62. **Lupo A**, **Coyne S**, **Berendonk TU**. 2012. Origin and evolution of antibiotic resistance: The common mechanisms of emergence and spread in water bodies. Front. Microbiol. **3**:1–13.

63. **Martínez JL**. 2008. Antibiotics and antibiotic resistance genes in natural environments. Science **321**:365–367.

64. **Baya AM**, **Brayton PR**, **Brown VL**, **Grimes DJ**, **Russek-Cohen E**, **Colwell RR**. 1986. Coincident plasmids and antimicrobial resistance in marine bacteria isolated from polluted and unpolluted Atlantic Ocean samples. Appl. Environ. Microbiol. **51**:1285–1292.

65. **Nair S**, **Chandramohan D**, **Loka Bharathi P a.** 1992. Differential sensitivity of pigmented and non-pigmented marine bacteria to metals and antibiotics. Water Res. **26**:431–434.

66. **Mudryk Z**, **Skórczewski P**. 1998. Antibiotic resistance in marine neustonic and planktonic bacteria isolated from the Gdansk Deep. Oceanologia **40**:125–136.

67. **Mudryk ZJ**. 2002. Antibiotic Resistance among Bacteria Inhabiting Surface and Subsurface Water Layers in Estuarine Lake Gardno. Polish J. Environ. Stud. **11**:401–406.

68. **Mudryk Z**, **Podgórska B**, **Ameryk A**. 2001. Bacteriological characterisation of a Baltic sandy beach in summer. Ecohydrol. Hydrobiol. **1**:503–509.

69. **Mudryk ZJ**. 2005. Occurrence and distribution antibiotic resistance of heterotrophic bacteria isolated from a marine beach. Mar. Pollut. Bull. **50**:80–86.

70. **Bogomolni AL**, **Gast RJ**, **Ellis JC**, **Dennett M**, **Pugliares KR**, **Lentell BJ**, **Moore MJ**. 2008. Victims or vectors: a survey of marine vertebrate zoonoses from coastal waters of the Northwest Atlantic. Dis. Aquat. Organ. **81**:13–38.

71. **Rose JM**, **Gast RJ**, **Bogomolni A**, **Ellis JC**, **Lentell BJ**, **Touhey K**, **Moore M**. 2009. Occurrence and patterns of antibiotic resistance in vertebrates off the Northeastern United States coast. FEMS Microbiol. Ecol. **67**:421–31.

72. **Blackburn JK**, **Mitchell MA**, **Blackburn MH**, **Curtis A**, **Thompson BA**. 2010. Evidence of Antibiotic Resistance in Free-Swimming , Top-Level Marine Predatory Fishes. J. Zoo Wildl. Med. **41**:7–16.

73. **Wallace CC**, **Yund PO**, **Ford TE**, **Matassa K a**, **Bass AL**. 2013. Increase in antimicrobial resistance in bacteria isolated from stranded marine mammals of the northwest atlantic. Ecohealth **10**:201–10.

74. **Smaldone G**, **Marrone R**, **Cappiello S**, **Martin G a**, **Oliva G**, **Cortesi ML**, **Anastasio A**. 2014. Occurrence of antibiotic resistance in bacteria isolated from seawater organisms caught in Campania Region: preliminary study. BMC Vet. Res. **10**:161.

75. **Cardoso de Oliveira AJF**, **Ranzani de França PT**, **Pinto AB**. 2009. Antimicrobial resistance of heterotrophic marine bacteria isolated from seawater and sands of recreational beaches with different organic pollution levels in southeastern Brazil: evidences of resistance dissemination. Environ. Monit. Assess. **169**:375–84.

76. **Cardoso de Oliveira AJF**, **Watanabe Pinhata JM**, **Pinhata JMW**. 2008. Antimicrobial resistance and species composition of Enterococcus spp. isolated from waters and sands of marine recreational beaches in Southeastern Brazil. Water Res. **42**:2242–2250.

77. **Dada AC**, **Ahmad A**, **Usup G**, **Heng LY**. 2013. Speciation and antimicrobial resistance of Enterococci isolated from recreational beaches in Malaysia. Environ. Monit. Assess. **185**:1583–99.

78. **Charnock C**, **Nordlie A-LL**, **Hjeltnes B**. 2014. Toxin Production and Antibiotic Resistances in Escherichia coli Isolated from Bathing Areas Along the Coastline of the Oslo Fjord. Curr. Microbiol. **69**:317–328.

79. **Thavasi R**, **Aparnadevi K**, **Jayalakshmi S**, **Balasubramanian T**. 2007. Plasmid mediated resistance in marine bacteria. J. Environ. Biol. **28**:617–621.

80. **Shaw KS**, **Rosenberg Goldstein RE**, **He X**, **Jacobs JM**, **Crump BC**, **Sapkota AR**. 2014. Antimicrobial susceptibility of *Vibrio vulnificus* and *Vibrio parahaemolyticus* recovered from recreational and commercial areas of Chesapeake Bay and Maryland Coastal Bays. PLoS One **9**:1–11.

81. **Mudryk ZJ**, **Kosiorek A**, **Perliński P**. 2013. In vitro antibiotic resistance of *Vibrio*-like organisms isolated from seawater and sand of marine recreation beach in the southern Baltic Sea. Hydrobiologia **702**:141–150.

82. **Alouache S**, **Kada M**, **Messai Y**, **Estepa V**, **Torres C**, **Bakour R**. 2012. Antibiotic Resistance and Extended-Spectrum β-Lactamases in Isolated Bacteria from Seawater of Algiers Beaches (Algeria). Microbes Environ. **27**:80–86.

83. **Nesme J**, **Cécillon S**, **Delmont TO**, **Monier JM**, **Vogel TM**, **Simonet P**. 2014. Large-scale metagenomic-based study of antibiotic resistance in the environment. Curr. Biol. **24**:1096–1100.

84. **Mudryk Z**, **Perliński P**, **Skórczewski P**. 2010. Detection of antibiotic resistant bacteria inhabiting the sand of non-recreational marine beach. Mar. Pollut. Bull. **60**:207–14.

85. **Hinrichsen D**. 1998. The Coastal Population Explosion. Next 25 Years Glob. Issues 27–29.

86. **Food and Agriculture Organization of the United Nations**. 2013. FAO Statistical Yearbook 2013: World Food and Agriculture. Rome.

87. **Poirel L**, **Liard A**, **Nordmann P**, **Mammeri H**. 2005. Origin of Plasmid-Mediated Quinolone Resistance Determinant QnrA Origin of Plasmid-Mediated Quinolone Resistance Determinant QnrA **49**:3523–3525.

88. **Leonard AFC**, **Zhang L**, **Balfour AJ**, **Garside R**, **Gaze WH**. 2015. Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. Environ. Int. **82**:92–100.

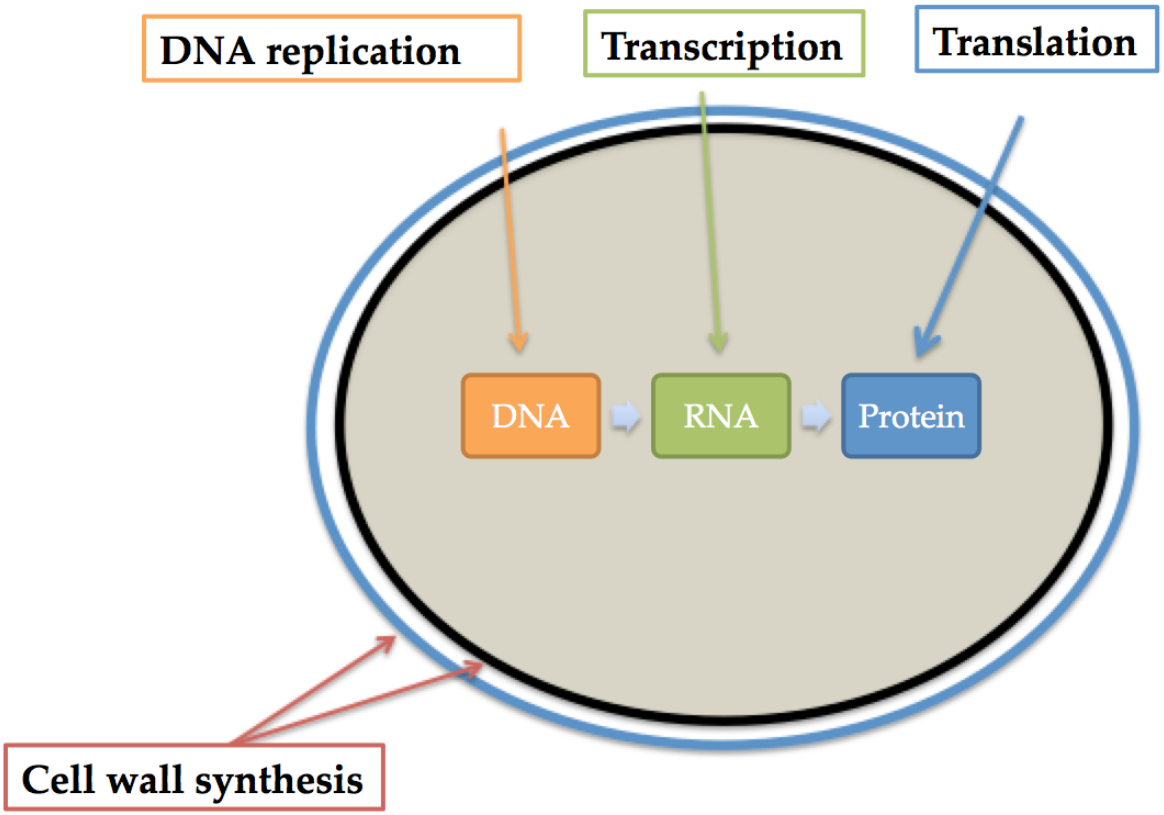

Figure 1. Illustration of antibiotic mechanisms.
Antibiotics have a wide variety of cellular targets. This diagram shows a few main mechanisms of antibiotic targets that relate to this dissertation. This diagram was modified from Lewis 2013 (6).

Figure 2. Illustration of antibiotic resistance in the marine environment.
Anthropogenic usage of antibiotics (white pills) in hospitals, homes, and agriculture make their way into the environment through wastes. In addition, bacteria (purple rod shaped) and antibiotic resistant bacteria (orange rods) can make their way into the environment. In the ocean, these anthropogenic derived antibiotics and bacteria can interact with the naturally present bacteria (teal circles), resistant bacteria (orange circles), and antibiotics.

CHAPTER 2: Caught Between the Clinic and the Environment Assessing Market and Political Failures, Knowledge, and Public Policy in Evaluating the Antibiotic Resistance Crisis

INTRODUCTION

Antibiotics can easily be considered one of the most important discoveries of the 20th century and have revolutionized the way healthcare treats bacterial infections. However, their immense power comes with a cost. This cost is antibiotic resistance (AR), a phenomenon that is present and rising throughout the world.

The extensive use of antibiotics has greatly contributed to the increase in prevalence of resistance. In 2013, Tom Friedlan, director of the Center for Disease Control (CDC), said, "If we're not careful, we will soon be in a post antibiotic era. And, in fact, for some patients and some microbes, we are already there (1)." If resistance continues to spread, this number could undoubtedly increase and humanity could advance to a point that antibiotics do not work for a majority of bacterial infections. In this scenario, even more lives would be impacted, as more serious illnesses that cannot be easily treated would become the norm. This change would not only affect those with bacterial infections, but any surgery or procedure requiring antibiotic as a preventive measure (i.e. cosmetic surgeries, hip replacements, chemotherapy) (2). The impacts of not having antibiotics to use would fundamentally change the medical advances the global community has made in the last fifty years (3, 4).

How have we gotten to this point? What has occurred that has allowed us to take something perceived to be "miracle" drugs to turning them into a public health crisis in less than a century? What scientific knowledge is currently known and what knowledge needs to be determined before taking action? What action, if any, should be taken? The goal of this chapter is to address these questions. The first section will report an analysis

of the market and political failures that have occurred that have increased antibiotic use, expanded antibiotic resistance, and failed to address the growing nature of resistance. The second section will focus on scientific knowledge of resistance in the clinical environment compared to the natural environment. The third section will address what remains to be acquired for greater analysis of antibiotic resistance and what evidence is necessary within each field to lead to appropriate action. The final section will end with recommendations for leaders in the field to combat antibiotic resistance.

What Went Wrong?: Market Failure in Addressing Antibiotic Resistance : Cost of Research and Development Unequal to Market Share

Numerous market and political failures have occurred in addressing antibiotics in the United States including unstable property rights, externalities, coordination problems, collective action, and organizational processes. Within the United States, policy decisions helping to rectify these issues have been relatively minimal. Market and political failures have exacerbated the resistance crisis and further action is necessary to amend these issues.

Research and development into pharmaceutical drugs is an extremely costly process with estimations of between $800 million to over two billion dollars to bring a new drug to market (5). To make an investment in research and development into drug candidates worthwhile, there has to be a market for these products. Antibiotics have intrinsically different characteristics that limit their market share; therefore, making antibiotics less profitable options for pharmaceutical research and development compared to other drugs (5). One of these characteristics is time scale. Antibiotics are used for short

time scales (days-weeks) and therefore require a relatively low number of treatments. This can be compared with products for chronic conditions such as depression, blood pressure, or diabetes that would be taken long term (months, years, decades) and would require a substantial number of treatments. Time scale can dramatically increase the market share. Therefore, an initial investment in a chronic medication would allow for a larger economic incentive compared to antibiotics, influencing pharmaceutical companies to products with higher economic payouts.

Another characteristic is that antibiotic discovery or invention requires even more investment and effort to discover new drugs compared to other pharmaceutical products. Brogan states that "high cost and significant technical effort" are necessary to discover new antibiotics, which further increases the price tag on antibiotic development (5). The increased cost makes it an even higher investment risk and a risker intervention.

Even if increased capital is available and if the technical barrier is achieved, a given broad-spectrum antibiotic would likely only be useful for a few years before the amount of resistance present makes it unfeasible to be prescribed. This phenomenon occurs due to the naturally short generation times of bacteria, allowing for resistance to be accumulated within a population. It is also amplified by the extreme amount of antibiotics utilized by humans (6). A way around this dilemma is to develop narrow spectrum antibiotics, which are antibiotics to be used for a specific type of bacteria. However, narrow spectrum antibiotics reduce the market share for that pharmaceutical. Yet, the antibiotic may be effective for a longer period of time than a broad-spectrum antibiotic, increasing the market share.

These economic issues point to the larger problem that more antibiotics and new targets will always be necessary as bacteria constantly evolve and change. In the words of Brogan, economic markets also fail for antibiotics "because the necessity for continual development of new antibiotics stems from the impending future threat of resistance, not just the current lack of efficacy" (5). Despite the necessity of antibiotics, these market failures have resulted in many pharmaceutical companies reducing or eliminating their antibiotic research, including Aventis, Bristol-Myers, Eli Lilly, and Proctor (7).

At this juncture, it seems that the market will not correct for failures. Economically, it does not benefit pharmaceutical companies to invest in antibiotic discovery because they will not be able to retrieve their initial (expensive) investments. To allow for discovery of antibiotics that are necessary to protect human health, it is urgent to correct these market failures by creating policy. One currently popular suggestion is for push-pull mechanisms with drug discovery and synthesis. Push incentives are to encourage research and development and involve giving initial investments to spur innovation (5, 8). This allows the cost (and therefore risk) to the industry completing the discovery process to be lower (5). Ways to achieve this would be to create funding from grants, adding tax breaks, or to increase the patent pool (5). The purpose of pull mechanisms are to increase the revenues for successful antibiotic development, further incentivizing pharmaceutical companies to invest in these products (5). These can include extended market exclusivity (8), other market guarantees (5), or prizes for establishment of a given drug (5). Spellberg et al. state that push incentives are likely

more beneficial compared to pull mechanisms because they allow for a smaller economic input, for the same value (8).

Obstacles to Addressing the Market Failures of Antibiotic Resistance

Market failures are not the only issue that has led to this resistance crisis. There have also been numerous political failures that have acted as obstacles towards obscuring solutions. This section will explore these failures, which include externalities, coordination problems, organizational processes, and collective action.

Mainly Negative Externalities Result from Resistance due to Antibiotic Use.

Use of antibiotics globally results in both positive and negative externalities, factors that affect individuals that they did not choose. One positive externality from antibiotic use is that proper use of antibiotics decreases the likelihood that the bacterial infection will be spread to others (9). If antibiotics were not used, the person with the illness could spread their infection to others in their community, generating a negative externality of disease that would then be shared to other individuals.

Sensitivity to antibiotics can be considered a natural resource—something that exists without human intervention as antibiotic sensitivity is a naturally occurring process. Despite the natural state of antibiotic sensitivity, this resource is affected by how we utilize it. In Hardin's Tragedy of the Commons, individual's self-interest overrides the best interest of the group (10). In the classic case, individuals would choose to put more and more sheep on the collective grazing land, allowing each individual to maximize their profits, even though it leads to the detriment of the resource (the grazing land), for the public (10). Antibiotic resistance can be examined as an

example of this framework. Individuals will use antibiotics as much as possible to maximize their benefits. For example, a farmer may feed his or her chickens with feed enhanced with antibiotics to promote growth and to reduce infections that may result from overcrowding (11). A patient may take more of an antibiotic than prescribed in order to feel better faster and to return to work. These individuals are acting in their own best self interest; however, these examples lead to increased overall resistance, which then erodes the natural resource creating a negative externality (12).

When antibiotics are used improperly—for viral infection instead of bacterial infection, when the full period of treatment is not followed, or when antibiotics are utilized for non-essential purposes (i.e. growth promoters in animals)—negative externalities are increased both locally and globally (9). These improper uses expand and increase antibiotic resistance in the world in a way beyond the control of both the market and private industry. Increased prevalence of resistance inflates the likelihood that an individual will become ill with a resistant infection. There will then be a rise in the cost of treatment for the patient, in terms of financial capabilities (more time in hospital, more expensive treatments), in terms of time (longer time away from work and family obligations), and in terms of health (reduced health, potential amputations, or even death in severe cases) (9, 13).

One solution to reduce these negative externalities is to price antibiotics using the "real" cost of antibiotics. Currently, most antibiotics are relatively inexpensive and one could argue that this results in misuse because the real cost is masked. The real cost should include loss of productivity and sick days to employers for employees who encounter resistant infections or higher taxes for Medicaid and Medicare for enhanced medical expenses due to a resistant

infection. Pigovian taxes, taxes to correct for negative externalities that result from inefficient markets, could be used to "levy a tax that equals the marginal external cost on consumption or production" (14). This additional economic cost has the potential to change behavior by forcing individuals to think through their actions. Instead of going to a physician's office and requesting an antibiotic for a viral infection, the cost could cause individuals to be willing to wait for a diagnostic test or to invest in over the counter remedies for the symptoms of their infection. Farmers may need to invest in more appropriate care for their animals instead of just routinely treating them with antibiotics in their feed to stimulate growth. The money collected from this tax could be used to fund antibiotic development (14), further helping the public health good.

There are two disadvantages of this Pigovian tax. One is that it is hard to determine the cost of resistance (14). The argument that we cannot completely determine resistance costs does not absolve us of the effort to work for rough estimations. The other disadvantage is that a tax may price out antibiotics as a treatment option for individuals in lower socioeconomic classes. This means that there would have to be an alternative mechanism to make these affordable for these individuals while still limiting widespread access to these goods. Despite these disadvantages, monetary costs of resistance as assessed with Pigovian taxes may be the incentive that allows for real change to be made to human use of antibiotics in a way that has not yet occurred through extensive education programs by public health programs across the globe.

Many Public and Private Stakeholders in the Process Increase Coordination Problems. Another failure is the coordination problems that occur due to the necessity of integration of different private and governmental institutions. In the United States, there are multiple stakeholders in the development and regulation of pharmaceuticals and their subsequent use in

the market. Predominantly private industry develops antibiotics, though some private pharmaceutical companies have partnered with laboratories in academia to complete screenings of potential targets (15). This requires coordination between research and academic laboratories to allow development of the most marketable products. To bring a product to market in the US, a private industry is required to go through a governmental approval and regulation process. The Food and Drug Administration (FDA) regulates and licenses pharmaceuticals and other goods used not only by us, but also animals, and requires a lengthy process of clinical trials that can last many years (16).

Not only does the US government have agencies that regulate drugs, in addition, there are many agencies that have an interest in how antibiotics are used. The Centers for Disease Control and Prevention (CDC) focuses on public health (mainly human) and they track resistance trends, spearhead campaigns to encourage prudent use of antibiotics, and work to protect human health. The CDC released a recent report on the threat of antibiotic resistance, calling attention to their growing problem (17). National Institutes of Health funds research related to discovering better treatments for antibiotic resistant infections (18).

Beyond these agencies for which antibiotic resistance is a pressing issue and a large part of their organization, there are also many agencies where their policies affect how we use antibiotics. Six agencies addressing concerns ranging from veteran affairs to Medicaid to healthcare research all participated in developing a plan for combatting antimicrobial resistance (19). All of the agencies mentioned work with human health. Their agency policies on antibiotics will impact how much and what kinds of resistance are developed within the United States and throughout the world. In terms of crops and food animal use of antibiotics, the

Department of Agriculture can set regulations on what farmers are allowed to use on their crops. The Environmental Protection Agency is charged with protection of the natural environment and how antibiotics affect the world around us. These examples show the extreme scope of governmental agencies interested in antibiotic use and the development of antibiotic resistance. This is further complicated by non-governmental organizations (pharmaceutical companies, environmental organizations, non-governmental organizations for the preservation of public health, medical facilities, etc.) that also would need to be included for effective policy and regulation.

Organizational Processes Unable to Adequately Cope with Antibiotic Resistance Crisis.

Not only are the many agencies a political and institutional failure in dealing with this crisis, but also the organizational processes contribute to failure. The governmental agencies work on relatively slow time scales, with their own bureaucratic interests and politics also being a factor (20). One specific example of an organizational failure is the substantial time and effort to go through the entire process with industry, FDA, and governmental agencies to bring a drug to market. This, coupled with the fact that drug discovery is itself slow and requires testing many, many more compounds than actually end up being successful, means that fruitful compounds do not make it to market for a number of years after they have been discovered or their efficacy for a given treatment have been determined. There have been attempts to hasten this process, but this can result in unsafe conditions for the consumers of these products with products that have not been sufficiently tested.

Collective Action Dilemma: How Rational Choice Limits Public Goods of Antibiotics.

Development of novel antibiotics to combat resistant infections can be considered an example of Mancur Olson's collective action dilemma (21). Having access to antibiotics that work benefits everyone in the entire world, regardless of if they expend effort or buy into the costs of research and development of these drugs. Essentially, this allows the majority of individuals to be "free riders" in having access to effective antibiotics to treat infections without paying the costs. The costs of these resources were previously borne by pharmaceutical companies, which represent a small group, compared with the all people that benefit from having antibiotics that work. Pharmaceutical companies were willing to take on this role because they gained economically from taking action, creating products that had a viable market that they could pursue. In contrast, many fewer pharmaceutical companies are completing this research now due to the lower economic incentives. This problem has the potential to impact every person. However, it would be nearly impossible for an individual, a group of individuals, or even one pharmaceutical company to take on this problem of their own accord. Not only would it be an irrational choice for an individual or a group in terms of the amount of energy and time expended, but also it would be difficult to gain the expertise and connections to interact with all the relevant agencies and companies. A solution to collective action problems is often that the government takes on providing (and regulating) a resource so thereby the energy and time is shared more equally between individuals. This is likely a necessary outcome for the antibiotic resistance case.

Conclusion of Market and Political Failures

Both market and political failures have had substantial impacts on the ability to address and begin to solve the crisis of antibiotic resistance. There has been a failure to create

appropriate economic incentives for development of new antibiotics, especially compared to more lucrative drugs. There are large, mainly negative externalities that result from the extensive use of antibiotics and further increase resistance in the world around us. These externalities result predominantly from the relatively easy access and inexpensive cost of antibiotics in the United States. However, antibiotic resistance is a global problem and affects high, medium, and low income countries. Coordination problems between numerous government agencies, slow moving and ill-suited organizational processes, and rational choice all further increase the problems of making any sort of action regarding antibiotic resistance. These problems are not the cause of one person or one institution, but instead indicate an interconnected issue. To address this global issue, knowledge assessment on the topic to lead to effective policy is necessary.

Importance of knowledge assessment

To make improved progress on the large-scale problem of antibiotic resistance market failures, effective policy is necessary. The market correcting itself is unlikely to happen, and individual actors do not have substantial power to impact this global problem. However, there is a large leap between market failures occurring and appropriate policy being created. This leap is knowledge assessment. Knowledge assessment refers to the ways we examine evidence, determine its reliability, and eventually use that knowledge to figure out if policy is needed and what aspects that policy may contain.

Knowledge assessment is not a trivial fight; it involves pointing out legitimate and illegitimate studies, facts, figures, and statistics. There are always downfalls with scientific studies—one could always obtain more samples, look in more locations for a given phenomenon, or have a longer time series. But, like in many things, scientists are limited

by time and funding. Furthermore, individuals (or organizations) can select scientific studies and knowledge that validate their opinions. We see this with climate change as individuals can select research that fits the conclusion or the decision they want to make. However, less nebulous processes can also occur. Information that might be important to determining a policy may be unknown, underfunded, or uncertain; thereby making it more difficult to assess what the correct scientific principles are and then, what policy may be most effective. Determining what knowledge to utilize in policy is crucial to addressing creation of effective and relevant recommendations.

In the case of antibiotic resistance, the critical issue is not if antibiotic resistance is a public health risk. This information is well established and accepted, even within disparate sectors (17, 22–25). The issue most prominent in the antibiotic resistance case is a failure to examine the entire process holistically, leading to extreme differences in knowledge between different sectors (e.g. greater amounts of research in the clinical environment compared to the natural environment) and a lack of integrated information leading to successful policy implementations. This section aims to uncover the process of knowledge assessment in addressing antibiotic resistance and provide recommendations of how to move towards a more holistic process.

Antibiotic Resistance is a Holistic Process involving both the Clinic and the Natural Environment.

Antibiotic resistance is a holistic process that involves inputs and outputs from various sectors of both natural and anthropogenic environments. A review paper by Davies and Davies illustrates the combined interactions of antibiotic and antibiotic resistance by cross cutting through various sectors in which we use antibiotics (agriculture,

wastewater, hospitals, communities) (6). Environmental resistance is critical to examine because pristine environments (with less impact from humans), hospitals, waste streams, and natural environments (soils, waters, animals, plants) are all linked together. A holistic approach recognizing that these aspects are linked, instead of viewing them as separate processes, would increase our knowledge and allow for approaches about policy that could be critical to impacting the world's ability to assess antibiotic resistance.

The environment represents a reservoir for antibiotic resistance (26–29). In fact, some of the clinically important resistances, such as class A extended spectrum beta-lactamase CTX-M gene and the quinolone resistance gene, are believed to have originated in the environment and transferred from the environment to humans, causing substantial health issues (30). Understanding environmental resistance can allow for mitigation of these transfers, increasing human safety and health and decreasing antibiotic resistance overall, especially in the clinic.

Explaining Why a Holistic Approach to Antibiotic Resistance Has Not Yet Been Taken.

Despite this knowledge that resistance is natural and exists in many environments, the manner that antibiotic resistance research is carried out has largely been completed in a sectored fashion that mirrors the way we think about the "natural" world and the "human" world. In general, the concept of pristine environments tends to conjure images of lakes, rivers, or remote regions like Antarctica or the Arctic, that are considered devoid of human impacts. Human environments such as cities, highways, and small towns are directly impacted by our processes. However, this dichotomy is not so well defined in reality. Human impacts go beyond our cities lines, past our highways, and further than

our individual country borders. Even what may be considered a pristine region can be impacted by human activities (31, 32).

Just as environments tend to be defined as "natural" or "human", the same occurs in antibiotic resistance research. The clinical environment can be described as areas of treatment for humans including a hospital, doctor's offices, rehabilitation centers, or nursing homes. Natural environments are the environments that exist beyond human created spaces. Humans can be affected by these environments and also affect these environments. These natural environments include water bodies such as lakes, rivers, streams, and the ocean, soil environments such as the beach or forests, wild animals such as deer, seals, and whales, and domesticated animals such as dogs, cattle, or chickens.

As might be expected, there are large differences between the knowledge accumulated about resistance in hospital environments compared to the natural environment. More funding, effort, and time has been spent focusing on antibiotic resistance within clinical areas. This is necessary and has helped dramatically reduce the spread of resistant infections in hospitals and has led to greater understandings of resistance dynamics. However, the issue is that this idea of resistance in hospitals tends to emphasize that resistance stops at hospital doors and does not move out into the natural environment. This idea has tended to reduce the importance of examining environmental resistance. Instead, the reality is that a resistant bacteria or resistance gene knows nothing of human created boundaries. This distinction between the clinical and natural environment is superficial at best. It certainly makes for more difficult studies to combine both the clinical and environmental sectors, especially when funding agencies and

scientists are often focused on only one of the sectors (EPA-on natural environment, NSF-on basic scientific questions, not health or environment related, NIH-on clinical environments). However, creating these false dichotomies between humans and nature only harms our ability to truly respond and mitigate the issues of antibiotic resistance.

Strategies to standardize testing antibiotic resistance in the natural environment.

The first hurdle to tackle is the definition of resistance itself. The operational definition of resistance is defined primarily in clinical ways, by how that bacteria would be affected by a given therapeutic treatment of the antibiotic. Currently, the procedure to analyze if a given bacterial isolate is resistant involves culturing that isolate and testing it to determine if the isolate is resistant or sensitive to the antibiotic(s) to be used for treatment of the infection (33, 34). This is very informative for choosing appropriate antibiotic treatment options. While this works in the clinical environment, it poses issues when it is transferred to environmental bacteria. One aspect is at what level of antibiotic resistance should environmental bacteria be categorized as resistant. Some resistances can be passed to other bacteria, including those that are pathogenic, showing how critical these impacts can be. One current option for this is proposed by Berendonk et al. 2015 and states that epidemiological cut-off values (ECOFF) be utilized instead. The ECOFF value looks at a given taxonomic grouping of bacteria and determines acquired resistance compared with populations that have no resistance (22). The ECOFF database values are relatively limited in terms of environmental bacteria, so this would require some concerted effort to expand the current database (22).

The next hurdle to overcome is how to appropriately assess resistance in a laboratory setting. Currently, disk diffusion or minimum inhibitory concentration methods are utilized to assess sensitivity or resistance to antibiotics. This occurs by growing the bacterial isolate, testing it in culture against discs embedded with antibiotics, and measuring the zones of inhibition, allowing for the assessment of resistance (CLSI method). This works very well for pathogenic clinical bacteria, which are well established in their growth patterns and knowledge of their resistances. This also allows clinical bacteria resistances to be compared over time and location, leading to a greater understanding of resistance trends and patterns (35, 36). However, this culturing process can be time-intensive and requires twenty four or more hours, which can be critical for treatment in some cases. Environmental bacteria do not as easily fall within this framework as it is thought that only 1% of all environmental bacteria are able to be cultured (37, 38). Therefore, culture-based methods leave the vast majority of bacteria unstudied- potentially hiding important insights into antibiotic resistance dynamics. Culture based AR techniques have been used in the environment (39–42), but in general, there is no consistent method to their application, leaving results difficult to compare between studies (22). Further, the exact procedures different researchers utilize are often not published in full detail, making it hard to replicate these techniques. To circumvent these culture-based application downfalls, non-culture based techniques, such as sequencing or polymerase chain reaction (PCR), may be utilized instead. However, this change in procedure may make it difficult to compare environmental results with the clinical

procedures that are already in place. Therefore, some way to compare these two divergent procedures would be necessary.

With an appropriate definition of resistance in place and useful standardized methods, global assessment of antibiotic resistance in the environment could be readily examined and compared between studies, locations, and regions. A standardized procedure would allow analysis of large-scale questions like: is antibiotic resistance in the environment increasing over time? Is environmental antibiotic resistance consistent over locations (i.e. are there "hotspots" for antibiotic resistance within or between countries)? What environments have the highest levels of antibiotic resistance? Is there a greater public health risk for interacting with one environment compared to another?

Delving deeply into the environment resistance would allow us to readily close the gap on questions regarding environmental resistance and better understand connections to the clinical environment. Analysis of many separate clinical data sources has been compiled and investigations of these larger data sets have been seen in the past few years in a variety of publications/open visualization sources from the Center for Disease Dynamics, Economics, and Policy and the World Health Organization (35, 36). These immense studies have allowed for analysis of changing resistance over time (36), resistance levels in different states (36), and resistance levels throughout the world (35, 36). If a standardized system is in place for the natural environment, similar levels of analysis could be completed. Then, the knowledge gained from the environment could be combined with the clinical knowledge that has already accumulated, hopefully leading to effective policy.

There is Enough Evidence and Scientific Consensus to Take Action on Public Policy.

At the crux of knowledge assessment is determining what action can or should be made from the given scientific research. In the case of antibiotic resistance, there are parts that are well established and action should be taken to make policy changes to impact human health. However, there are other environmental sectors that require increased research. Despite the need for increased research for the natural environment, the knowledge already obtained from the clinical environment and the overall impacts of the substantial use of antibiotics by humans illustrate enough evidence and consensus to create public policy and action.

Some actions are already being taken both from research obtained in the clinical and environmental fields. Certainly in the clinical realm, there is a known desire to reduce the amount of antibiotics being used by the general public. Many campaigns by the CDC and other governmental and non-governmental agencies throughout the world have tried to focus public attention on why antibiotics are only useful in the case of a bacterial infection, helping to reduce resistance by reducing improper use of antibiotics. In the environment, it is relatively well established that use of antibiotics in agriculture increases resistance (43). Actions are being taken to reduce the amount of antibiotics in this sector by major agricultural producers (Perdue) and first level large-scale consumers (McDonalds, Chipotle, Panera) due predominantly to consumer demand and interest (44). These large-scale consumers can have a heightened impact due to their large amount of consumption, allowing them to force producers into adopting their policies because otherwise the producers risk losing a large buyer. Consumer demand for antibiotic-free products may in turn drive restaurants and grocery stores to require antibiotic-free items,

thereby further encouraging producers to adopt these policies. However, optional procedures and campaigns will likely not contain enough power to fully address the scale of this global problem.

More research needs to be completed to fully understand the full impacts of environmental antibiotic resistance. However, the main actions to be taken for antibiotic use are reducing consumption of antibiotics and increasing novel treatment options for resistant infections. These actions are known to be effective from other sectors (i.e. clinical and environmental research that has already been completed). In this light, there is no reason not to act on the policies and treatment that are known to be effective. Research on lesser-known issues can be continued. It will likely take substantial time and effort to implement procedures and policies on reducing consumption and increasing novel treatment, so working on both sectors of taking action and continuing research is necessary.

For matters that are relatively unknown, further research needs to be undertaken before action is taken. For example, evidence suggests that wastewater treatment plants serve as hotspots of antibiotic resistance transfer because of the various waste inputs from many diverse locations that contain antibiotics, antibiotic resistance genes, and antibiotic resistant bacteria (45–48). All of these inputs along with the process of wastewater treatment provide selective pressures—increasing gene transfer or selecting for resistance. Increased research on intervention possibilities would need to be done to find a relevant technological change and then policy should be utilized to enact the best possible manipulation.

Meeting the Needs of Key Players in the Antibiotic Resistance Crisis.

Examination of those who utilize antibiotics should first be completed and include the general public and farmers of fish, meat, and plants. The general public often wants fast fixes to their illnesses in order to feel better sooner and to get back to work and to their lives. A competing factor at play here is that much of the general public in the United States does not understand how antibiotics work and that antibiotics are only effective for bacterial infections. Due to their large number, the general public does have a large amount of power in terms of economic capital. If they are able to organize, which is often difficult due to diffuse interests (Olson), they can make change. For example, use of antibiotic treated food in certain restaurants has decreased in part to consumer demands (44).

There are also key players in the United States in prescribing antibiotics. These individuals often act as the gatekeepers between antibiotic production and the general public. These include predominantly physicians/clinicians and veterinarians (for therapeutic or sub-therapeutic uses of antibiotics for animals). The need for a prescription leaves clinicians with two opposing ideas: 1.) desire to care for their patients and a need to maintain their client base and 2.) knowledge that use of antibiotics for inappropriate conditions increases resistance. A patient may want an antibiotic and may go to another doctor or veterinarian if the patient deems they were treated inappropriately or unfairly by not getting what, in their eyes, is the appropriate treatment. These practitioners essentially are forced to choose between patient satisfaction or being scientifically correct and limiting resistance from developing.

Another key player in terms of access to antibiotics are makers of animal feed, which can often contain antibiotics. For animals, the FDA has instituted a collaborative (voluntary) approach to reduce the use of antibiotics in animal feed and to only use antibiotics for therapeutic purposes instead of growth promotion. However, for the food producers and antibiotic producers- this would reduce their market share, leading to lower financial gains for their products. It seems unlikely that this voluntary approach will be successful, but only time will tell.

As illustrated in this section, there are a diverse group of stakeholders in this topic and their needs are varied. This makes it difficult to create policy because it is impossible to have policies that are going to be appropriate for everyone's needs. However, these burdensome realities should not be used as an excuse to avoid creating policy. Instead, there should be an acknowledgement of winners and losers within given policies. Value judgments regarding the importance of various sectors must be made in creating these policies as well. With these factors in mind, recommendations for where to go and how to move forward are discussed below.

What scientific and policy evidence recommendations exist for these fields?

Recommendations for the Clinical Field

Recommendation 1: Acknowledge and examine the interdisciplinary framework of antibiotic resistance.

Recommendation 2: Research the intersections of the clinical field and the environment to determine what effects these may have.

Recommendations for the Environmental Field

Recommendation 1: Acknowledge and understand the interdisciplinary framework this research fits into.

Recommendation 2: As discussed earlier, creation of standardized methods, which are:

- able to be compared between both fields (clinical and the environment)
- relatively inexpensive (in terms of equipment usage as well as on a per sample basis)
- do not require advanced machinery (so as to be readily available for a greater number of locations)
- able to be compared with the data already existing from the disk diffusion method used predominantly by the clinical realm.

New methods (such as sequencing) or indicator organisms for an environment may prove helpful in this recommendation.

Recommendation 3: Increase sectors that are involved in testing. What other environments are important and need to be studied? What incentives can be placed to examine these regions? Should a collective group of experts determine priorities along with funding agencies?

Collective Recommendations for Both Clinical and Environmental Groups Interested in Antibiotic Resistance

Recommendation 1: Create methods the evaluate risk of antibiotic resistance within a given environment. Port et al. 2014 has a method to evaluate risk using community composition, gene transfer potential, antibiotic resistance gene potential, and pathogenicity potential (49, 50).

Recommendation 2: Collaborate with others' expertise to answer interdisciplinary questions. Clinical microbiologists and environmental microbiologists should work together to answer scientific questions relating to antibiotic resistance. These fields are interconnected and that the only way to control antibiotic resistance is with a collaborative approach (22).

Recommendation 3: Create ways to disseminate results so that both communities are aware of the conclusions. Currently, it seems that results are fragmented—environmental microbiologists publish in journals such as Marine Pollution Bulletin (51), Applied and Environmental Microbiology (52–54), or Environmental Science and Technology (55–57) whereas clinical microbiologists publish in journals such as Clinical Infectious Diseases (58–60), Expert Opinion on Pharmacotherapy (61), Clinical Microbiology and Infection (62), or Clinical Microbiology Reviews (63). This leads researchers to fail to find results and collaborations that could be very relevant to their work.

A solution to this would be encourage an interdisciplinary journal or alternatively, to encourage (in tenure packages or through funding agencies) dissemination of antibiotic resistance work across other relevant fields. Creation of a specific interdisciplinary conference of clinical and environmental microbiologists interested in antibiotic resistance could also be extremely relevant and useful to addressing these problems.

General Policy Recommendations for the United States

General Recommendation 1: Reduce use of antibiotics

1.) Incentives to hospitals to reduce use. One way to reduce use of antibiotics in hospitals could be to provide incentives to hospitals to reduce their use. There are a variety of ways

that this has been proposed including allocation of antibiotic prescriptions per hospital or per doctor (64) and docking funding reimbursements by the government if resistant infections are rampant (some of these policies already exist for hospital acquired conditions with Medicare) (65, 66). This is a somewhat problematic idea as it is difficult to force individuals to complete the entire dose of their prescription and it may not be fair to tie reimbursements to individuals' actions. However, having financial incentives often spurs individuals to take action so perhaps this is a relevant and useful way to proceed.

2.) Reduce use in food production. The United States uses significantly more antibiotics in animal production than for humans- roughly three times more (67). Work has been in progress to reduce the amount of antibiotics in agriculture and aquaculture and this work is continuing and should likely be expanded (68–71). Innovation will likely be a critical tool in determining new methods for food production with reduced antibiotics.

Passing legislation so that it is illegal to use antibiotics in food products for growth production would reduce use. Though, of course, there would be loopholes around this legislation- individuals could state that they are using the antibiotics for disease prevention or disease treatment when really they are using it for growth promotion. Perhaps a way around the issue of continuing to use antibiotics for their growth promotion capabilities might be to require oversight as to how often antibiotics are being utilized on larger farms. This legislation would be difficult to initially pass as many producers would be against it, but it has been successful in other countries (Denmark for example) (72, 73) and could greatly reduce use of antibiotics.

It is possible that this legislation would spur innovation and force food producers to increase efficiencies as the Porter Hypothesis would predict (74, 75). Part of the reason producers use subtherapeutic levels of antibiotics is to control infection because of the densely populated farms where infection can run rampant. Legislating antibiotic use in food production could create efficiencies to care for those animals in a more sustainable manner. If this legislation is passed and prices increase, there may be a need to provide subsidies to individuals in lower socioeconomic classes for the increase cost of meat and other food products (eggs, cheese).

3.) Increase science education. A large reason that individuals request antibiotics for illnesses is because they do not understand the differences between bacterial versus viral infections. Informal and formal education to increase scientific literacy would help to address this lack of knowledge.

4.) Create incentives for rapid diagnostics. Rapid diagnostics for testing of individuals' infections for the type of infection (bacterial, viral, fungal, etc.) would be critical to determining appropriate treatment, especially for individuals who are severely ill. Further, the diagnostic could then determine to what antibiotics the infectious agent might show resistance (76–78). Overall, this would allow for a more efficient way of utilizing antibiotics. Subsidies or incentives may be necessary to create these diagnostics and subsidies may also be necessary to ensure they are utilized in hospitals.

General Recommendation 2: Increase amount of new antibiotics

1.) Provide incentives for companies to take on antibiotic development. As discussed in the first half of this paper, there are not adequate market incentives for companies to take

on drug development. This means that the government either needs to designate an agency for this task or incentivize companies to take this on themselves. This is a potential downfall because it is another factor that government and bureaucracy has control of, which may not lead to the most efficient process.

2.) Provide research grants to academic laboratories to screen for new antibiotics. Often in drug development, academic laboratories screen many promising products and then these products are sold to pharmaceutical companies if they are found to be effective. Further grant money could be provided to academic laboratories to screen for specific types of antibiotics that are effective against certain bacteria. It may be wise to incentivize screening from the natural environment, since that is where the majority of antibiotics come from and where there is an abundance of untapped potential resources.

Recommendation 3: Reduce resistance and infections

1.) Create and increase monitoring programs for resistant infections. Some infections are already monitored by the CDC (17) and include : *Streptococcus pneumoniae*, Methicillin-resistant *Staphylococcus aureus*, *Salmonella*, *Enterococcus*, and *Mycobacterium tuberculosis*(17). However, a larger resource would be helpful to track infections and to better understand the dynamics of these infections. A global registry would be phenomenal, but would require much undertaking, especially for unstable or undeveloped regions (35, 79, 80). However, with the ease of travel in our global world (81), knowledge of antibiotic resistance threats are critical to global preparedness.

2.) Increase research into the environment. The environment is likely a source of resistance to be passed to our food products and ourselves when we interact with the

environment (54, 82, 83). Understanding resistance transfer in the environment could allow minimization of resistance. Additionally, if there are hotspots that are found to contain large amount of resistance, monitoring of these areas could occur to reduce human interaction to decrease resistance transfer. Risk assessments and calculations would be necessary as well. To do this research appropriately, interdisciplinary research between clinical researchers and environmental scientists is necessary. Creating specific funding pools for this research could incentivize this.

CONCLUSION

This chapter has focused on the issues associated with antibiotic resistance within the United States. However, antibiotic resistance is a global problem that needs global solutions (3, 23, 76, 84). The boundaries of antibiotic resistant bacteria are limitless. Human defined boundaries such as countries are not taken into account at all by bacteria and the ever more rapid modes of transportation only increase the reality of transfer between distant countries (81). How the global community acts on this pressing issue is critical to the health of humans and the environment both now and in the future. The antibiotic pipeline is time consuming and is not something that can be discovered overnight. Action now is critical to ensuring that we do not return to a pre-antibiotic time period, which would significantly detract from our medical advances in the past fifty years and our ability to continue making improvements medically, technologically, and societally in the world.

References

1. **Centers for Disease Control and Prevention**. 2013. Press Briefing Transcript. CDC.
2. **White AR**. 2011. Effective antibacterials: at what cost? The economics of antibacterial resistance and its control. J. Antimicrob. Chemother. **66**:1948–53.
3. **Laxminarayan R**, **Matsoso P**, **Pant S**, **Brower C**, **Røttingen J**, **Klugman K**, **Davies S**. 2015. Access to effective antimicrobials: a worldwide challenge. Lancet **151**:1–8.
4. **Nathan C**, **Carrs O**. 2014. Antibiotic Resistance- Problems, Progress, and Prospects. Perspective **371**:1761–1763.
5. **Brogan DM**, **Mossialos E**. 2013. Incentives for new antibiotics: the Options Market for Antibiotics (OMA) model. Global. Health **9**:1–10.
6. **Davies J**, **Davies D**. 2010. Origins and Evolution of Antibiotic Resistance. Microbiol. Mol. Biol. Rev. **74**:417–433.
7. **Projan SJ**. 2003. Why is big Pharma getting out of antibacterial drug discovery? Curr. Opin. Microbiol. **6**:427–430.
8. **Spellberg B**, **Sharma P**, **Rex JH**. 2012. The critical impact of time discounting on economic incentives to overcome the antibiotic market failure. Nat. Rev. Drug Discov. **11**:168–168.
9. **Eggleston K**, **Zhang R**, **Zeckhauser RJ**. 2010. The Global Challenge of Antimicrobial Resistance: Insights from Economic Analysis. Int. J. Environ. Res. Public Health **7**:3141–3149.
10. **Hardin G**. 1968. The Tragedy of the Commons. Science (80-.). **162**:1243–1248.
11. **Ghosh S**, **LaPara TM**. 2007. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J. **1**:191–203.
12. **Cully M**. 2014. The politics of antibiotics. Nature **509**:S16–S17.
13. **Coast J**, **Smith RD**, **Millar MR**. 1998. An economic perspective on policy to reduce antimicrobial resistance. Soc. Sci. Med. **46**:29–38.
14. **Vågsholm I**, **Höjgård S**. 2010. Antimicrobial sensitivity-A natural resource to be protected by a Pigouvian tax? Prev. Vet. Med. **96**:9–18.
15. **Lewis K**. 2013. Platforms for antibiotic discovery. Nat. Rev. Drug Discov. **12**:371–87.
16. **Eichler H-G**, **Oye K**, **Baird LG**, **Abadie E**, **Brown J**, **L Drum C**, **Ferguson J**, **Garner S**, **Honig P**, **Hukkelhoven M**, **Lim JCW**, **Lim R**, **Lumpkin MM**, **Neil G**, **O'Rourke B**, **Pezalla E**, **Shoda D**, **Seyfert-Margolis V**, **Sigal E V**, **Sobotka J**, **Tan D**, **Unger TF**, **Hirsch G**. 2012. Adaptive Licensing: Taking the Next Step in the Evolution of Drug Approval. Clin. Pharmacol. Ther. **91**:426–437.
17. **Center for Disease Control and Prevention**. 2013. Antibiotic resistance threats in the United States, 2013.
18. **National Institute of Allergy and Infectious Diseases**. 2015. National Institute of Allergy and Infectious Diseases. NIAID website.
19. **Centers for Disease Control and Prevention**. 2012. A Public Health Action Plan to Combat Antimicrobial ResistanceInteragency Task Force on Antimicrobial Resistance.
20. **Allison GT**. 1969. Conceptual Models and the Cuban Missile Crisis **64**:689–718.

21. **Olson M**. 1971. The Logic of Collective Action: Public Goods and the Theory of Groups. Harvard University Press, Cambridge.
22. **Berendonk TU**, **Manaia CM**, **Merlin C**, **Fatta-Kassinos D**, **Cytryn E**, **Walsh F**, **Bürgmann H**, **Sørum H**, **Norström M**, **Pons M-N**, **Kreuzinger N**, **Huovinen P**, **Stefani S**, **Schwartz T**, **Kisand V**, **Baquero F**, **Martinez JL**. 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 1–8.
23. **Carlet J**, **Pulcini C**, **Piddock LJ V**. 2014. Antibiotic resistance: a geopolitical issue. Clin. Microbiol. Infect. **20**:949–53.
24. **Shallcross LJ**, **Howard SJ**, **Fowler T**, **Davies SC**. 2015. Tackling the threat of antimicrobial resistance: from policy to sustainable action. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **370**:20140082-.
25. **Marti E**, **Variatza E**, **Balcazar JL**. 2014. The role of aquatic ecosystems as reservoirs of antibiotic resistance. Trends Microbiol. **22**:36–41.
26. **Cantas L**, **Shah SQ a**, **Cavaco LM**, **Manaia CM**, **Walsh F**, **Popowska M**, **Garelick H**, **Bürgmann H**, **Sørum H**. 2013. A brief multi-disciplinary review on antimicrobial resistance in medicine and its linkage to the global environmental microbiota. Front. Microbiol. **4**:1–14.
27. **Finley RL**, **Collignon P**, **Larsson DGJ**, **Mcewen SA**, **Li XZ**, **Gaze WH**, **Reid-Smith R**, **Timinouni M**, **Graham DW**, **Topp E**. 2013. The scourge of antibiotic resistance: The important role of the environment. Clin. Infect. Dis. **57**:704–710.
28. **Pruden A**, **Larsson DGJ**, **Amézquita A**, **Collignon P**, **Brandt KK**, **Graham DW**, **Lazorchak JM**, **Suzuki S**, **Silley P**, **Snape JR**, **Topp E**, **Zhang T**, **Zhu Y-G**. 2013. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. Environ. Health Perspect. **121**:878–85.
29. **Ashbolt NJ**, **Amézquita A**, **Backhaus T**, **Borriello P**, **Brandt KK**. 2013. Human Health Risk Assessment (HHRA) for Environmental Development and Transfer of Antibiotic Resistance. Environ. Health Perspect. **121**:993–1002.
30. **Perry J a.**, **Wright GD**. 2013. The antibiotic resistance "mobilome": searching for the link between environment and clinic. Front. Microbiol. **4**:1–7.
31. **Bhullar K**, **Waglechner N**, **Pawlowski A**, **Koteva K**, **Banks ED**, **Johnston MD**, **Barton H a**, **Wright GD**. 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS One **7**:e34953.
32. **Clemente JC**, **Pehrsson EC**, **Blaser MJ**, **Sandhu K**, **Gao Z**, **Wang B**, **Magris M**, **Hidalgo G**, **Contreras M**, **Noya-Alarcon O**, **Lander O**, **McDonald J**, **Cox M**, **Walter J**, **Oh PL**, **Ruiz JF**, **Rodriguez S**, **Shen N**, **Song SJ**, **Metcalf J**, **Knight R**, **Dantas G**, **Dominguez-Bello MG**. 2015. The microbiome of uncontacted Amerindians. Sci. Adv. **1**:e1500183–e1500183.
33. **Clinical and Laboratory Standards Institute**. 2012. Performance Standards for Antimicrobial Disk Susceptibility Tests, 11th ed. Wayne, PA.
34. **Bauer AW**, **Kirby WM.**, **Sherris JC**, **Turck M**. 1966. Antibiotic Susceptibility by a Standardized Single Disk Method. Am J Clin Pathol **45**:493–6.
35. **World Health Organization**. 2014. Antimicrobial Resistance: Global Report on surveillance.
36. **White A**. 2015. CDDEP Maps Dangerous Trends in Antibiotic Ressitance on a

Global Scale for the First Time. Washington D.C.
37. **Staley JT**, **Konopka a**. 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. **39**:321–346.
38. **Walsh F**, **Duffy B**. 2013. The culturable soil antibiotic resistome: a community of multi-drug resistant bacteria. PLoS One **8**:e65567.
39. **Mudryk ZJ**, **Kosiorek A**, **Perliński P**. 2013. In vitro antibiotic resistance of *Vibrio*-like organisms isolated from seawater and sand of marine recreation beach in the southern Baltic Sea. Hydrobiologia **702**:141–150.
40. **Dada AC**, **Ahmad A**, **Usup G**, **Heng LY**. 2013. Speciation and antimicrobial resistance of Enterococci isolated from recreational beaches in Malaysia. Environ. Monit. Assess. **185**:1583–99.
41. **Mudryk Z**, **Perliński P**, **Skórczewski P**. 2010. Detection of antibiotic resistant bacteria inhabiting the sand of non-recreational marine beach. Mar. Pollut. Bull. **60**:207–14.
42. **Levin-Edens E**, **Soge OO**, **No D**, **Stiffarm A**, **Meschke JS**, **Roberts MC**. 2012. Methicillin-resistant Staphylococcus aureus from Northwest marine and freshwater recreational beaches. FEMS Microbiol. Ecol. **79**:412–20.
43. **Marshall BM**, **Levy SB**. 2011. Food animals and antimicrobials: Impacts on human health. Clin. Microbiol. Rev. **24**:718–33.
44. **Kesmodel D**, **Bunge J**, **McKay B**. 2014. Meat Companies Go Antibiotics-Free as More Consumers Demand It. Wall Str. J.
45. **Zhang H**, **Liu P**, **Feng Y**, **Yang F**. 2013. Fate of antibiotics during wastewater treatment and antibiotic distribution in the effluent-receiving waters of the Yellow Sea, northern China. Mar. Pollut. Bull.
46. **Gao P**, **Munir M**, **Xagoraraki I**. 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. Sci. Total Environ. **421**–**422**:173–83.
47. **Rizzo L**, **Manaia C**, **Merlin C**, **Schwartz T**, **Dagot C**, **Ploy MC**, **Michael I**, **Fatta-Kassinos D**. 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. **447**:345–360.
48. **Bouki C**, **Venieri D**, **Diamadopoulos E**. 2013. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: A review. Ecotoxicol. Environ. Saf. **91**:1–9.
49. **Port JA**. 2012. Public health applications of gene-environment interactions for Oceans and Human Health. University of Washington.
50. **Port JA**, **Cullen ACAC**, **Wallace JC**, **Smith MN**, **Faustman EM**. 2014. Metagenomic frameworks for monitoring antibiotic resistance in aquatic environments. Environ. Health Perspect. **122**:222–228.
51. **Vignesh S**, **Muthukumar K**, **James RA**, **Arthur James R**. 2012. Antibiotic resistant pathogens versus human impacts: a study from three eco-regions of the Chennai coast, southern India. Mar. Pollut. Bull. **64**:790–800.

52. **Parveen S**, **Murphree RL**, **Edmiston L**, **Kaspar CW**, **Portier KM**, **Tamplin ML**. 1997. Association of multiple-antibiotic-resistance profiles with point and nonpoint sources of Escherichia coli in Apalachicola Bay. Appl. Environ. Microbiol. **63**:2607–12.
53. **Jindal A**, **Kocherginskaya S**, **Mehboob A**, **Robert M**, **Mackie RI**, **Raskin L**, **Zilles JL**. 2006. Antimicrobial use and resistance in swine waste treatment systems. Appl. Environ. Microbiol. **72**:7813–20.
54. **Zurek L**, **Ghosh A**. 2014. Insects Represent a Link between Food Animal Farms and the Urban Environment for Antibiotic Resistance Traits. Appl. Environ. Microbiol. **80**:3562–3567.
55. **Pruden A**, **Arabi M**, **Storteboom HN**. 2012. Correlation between upstream human activities and riverine antibiotic resistance genes. Environ. Sci. Technol. **46**:11541–9.
56. **Graham DW**, **Olivares-Rieumont S**, **Knapp CW**, **Lima L**, **Werner D**, **Bowen E**. 2011. Antibiotic resistance gene abundances associated with waste discharges to the Almendares river near Havana, Cuba. Environ. Sci. Technol. **45**:418–424.
57. **Shah SQ a**, **Colquhoun DJ**, **Nikuli HL**, **Sørum H**. 2012. Prevalence of antibiotic resistance genes in the bacterial flora of integrated fish farming environments of Pakistan and Tanzania. Environ. Sci. Technol. **46**:8672–9.
58. **Spellberg B**, **Guidos R**, **Gilbert D**, **Bradley J**, **Boucher HW**, **Scheld WM**, **Bartlett JG**, **Edwards Jr. J**, **Edwards J**. 2008. The epidemic of antibiotic-resistant infections: a call to action for the medical community from the Infectious Diseases Society of America. Clin. Infect. Dis. **46**:155–64.
59. **Bartlett JG**, **Gilbert DN**, **Spellberg B**. 2013. Seven ways to preserve the Miracle of antibiotics. Clin. Infect. Dis. **56**:1445–1450.
60. **Jorgensen JH**, **Ferraro MJ**. 2009. Antimicrobial susceptibility testing: a review of general principles and contemporary practices. Clin. Infect. Dis. **49**:1749–55.
61. **Barriere SL**. 2015. Clinical, economic and societal impact of antibiotic resistance. Expert Opin. Pharmacother. **16**:151–153.
62. **Cantón R**. 2009. Antibiotic resistance genes from the environment: a perspective through newly identified antibiotic resistance mechanisms in the clinical setting. Clin. Microbiol. Infect. **15 Suppl 1**:20–25.
63. **Mundy LM**, **Sahm DF**, **Gilmore M**. 2000. Relationships between Enterococcal Virulence and Antimicrobial Resistance. Clin. Microbiol. Rev. **13**:513–522.
64. **Sage WM**, **Hyman DA**. Combating Antimicrobial Resistance: Regulatory Strategies and Institutional Capacity 1–38.
65. **Conway P**. 2013. U.S. Efforts to Reduce Healthcare-Associated Infections. Washington D.C.
66. **President's Council of Advisors on Science and Technology**. 2014. Report to the President on Combating Antibiotic Resistance.
67. **The Pew Charitable Trusts**. 2013. Record-High Antibiotic Sales for Meat and Poultry Production. Pew Charit. Trust. Web Page.
68. **Tilman D**, **Cassman KG**, **Matson PA**, **Naylor R**, **Polasky S**. 2002. Agricultural sustainability and intensive production practices. Nature **418**.

69. **Kumar Sahu M**, **Swarnakumar SNS**, **Thangaradjou KST**. 2008. Probiotics in aquaculture : importance and future perspectives. Indian J. Microbiol 299–308.
70. **Balcázar JL**, **de Blas I**, **Ruiz-Zarzuela I**, **Cunningham D**, **Vendrell D**, **Múzquiz JL**. 2006. The role of probiotics in aquaculture. Vet. Microbiol. **114**:173–186.
71. **Horrigan L**, **Lawrence RS**, **Walker P**. 2002. How Sustainable Agriculture Can Address the Environmental and Human Health Harms of Industrial Agriculture. Environ. Health Perspect. **110**:445–456.
72. **Levy S**. 2014. Reduced Antibiotic Use in Livestock: How Denmark Tacked Resistance. Environ. Health Perspect. **122**:160–165.
73. **Wielinga PR**, **Jensen VF**, **Aarestrup FM**, **Schlundt J**. 2014. Evidence-based policy for controlling antimicrobial resistance in the food chain in Denmark. Food Control **40**:185–192.
74. **Mohr RD**, **Saha S**. 2008. Distribution of Environmental Costs and Benefits , Additional Distortions , and the Porter Hypothesis. Land Econ. **84**:689–700.
75. **Ambec S**, **Cohen MA**, **Elgie S**, **Lanoie P**. 2013. The Porter Hypothesis at 20 : Can Environmental Regulation Enhance Innovation and Competitiveness ? **7**:2–22.
76. **Laxminarayan R**, **Duse A**, **Wattal C**, **Zaidi AKM**, **Wertheim HFL**, **Sumpradit N**, **Vlieghe E**, **Hara GL**, **Gould IM**, **Goossens H**, **Greko C**, **So AD**, **Bigdeli M**, **Tomson G**, **Woodhouse W**, **Ombaka E**, **Peralta AQ**. 2013. Antibiotic resistance — the need for global solutions. Lancet Infect. Dis. **3099**:1057--1098.
77. **Holmes AH**, **Moore LSP**, **Sundsfjord A**, **Steinbakk M**, **Regmi S**, **Karkey A**, **Guerin PJ**, **Piddock LJ V**. 2015. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet **6736**.
78. **Mendelson M**, **Balasegaram M**, **Gopinathan U**, **Hamer DH**, **Wertheim H**, **Basnyat B**, **Butler C**, **Tomson G**. 2015. Maximising access whilst curbing excess: achieving appropriate human antimicrobial use in low and middle income countries. Lancet **6736**:[under review].
79. **Okeke IN**, **Laxminarayan R**, **Bhutta Z a**, **Duse AG**, **Jenkins P**, **O'Brien TF**, **Pablos-Mendez A**, **Klugman KP**. 2005. Antimicrobial resistance in developing countries. Part I: recent trends and current status. Lancet Infect. Dis. **5**:481–493.
80. **Okeke IN**, **Klugman KP**, **Bhutta Z a.**, **Duse AG**, **Jenkins P**, **O'Brien TF**, **Pablos-Mendez A**, **Laxminarayan R**. 2005. Antimicrobial resistance in developing countries. Part II: Strategies for containment. Lancet Infect. Dis. **5**:568–580.
81. **Nordahl Petersen T**, **Rasmussen S**, **Hasman H**, **Carøe C**, **Bælum J**, **Charlotte Schultz A**, **Bergmark L**, **Svendsen CA**, **Lund O**, **Sicheritz-Pontén T**, **Aarestrup FM**. 2015. Meta-genomic analysis of toilet waste from long distance flights; a step towards global surveillance of infectious diseases and antimicrobial resistance. Sci. Rep. **5**:11444.
82. **González-Zorn B**, **Escudero J a.** 2012. Ecology of antimicrobial resistance: Humans, animals, food and environment. Int. Microbiol. **15**:101–109.
83. **Silbergeld EK**, **Graham J**, **Price LB**. 2008. Industrial food animal production, antimicrobial resistance, and human health. Annu. Rev. Public Health **29**:151–69.
84. **Sengupta S**, **Chattopadhyay MK**. 2012. Antibiotic Resistance of Bacteria : A Global Challenge 177–192.

CHAPTER 3 Antibiotic resistance in *Vibrio*-like bacteria is common at marine beaches on Cape Cod, MA

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ABSTRACT

Antibiotic resistance (AR) is a natural process, enhanced by anthropogenic antibiotic use. Natural environments, like the ocean, act as reservoirs of resistance, but until recently little research has examined their dynamics. Six beaches on Cape Cod, MA, with varying human impacts, were sampled over one year on nine occasions. *Vibrio*-like bacteria were isolated from wet sand, dry sand, and water from each beach and tested for sensitivity to five antibiotics (amoxicillin/clavulanic acid, ciprofloxacin, doxycycline, oxytetracycline, and trimethoprim) using the disk diffusion method. 73% of isolates showed resistance to at least one antibiotic, and resistance was persistent over time, space, and sample type. Isolates commonly exhibited trimethoprim, ciprofloxacin, and/or amoxicillin resistance. 16S ribosomal DNA amplicon-based community structure varied along with the dominant operational taxonomic unit (OTU). Permutational multivariate analysis of variance (PERMANOVA) indicate that resistance patterns, prevalence, and bacterial community composition were often related to month of sampling. Seasonal environmental variables also explain AR and community structure data. Distance based linear models (DistLM) using arcGIS land use variables reflect homogeneity in land use between sites. Estimates of *Vibrio*-like resistant bacteria range from 57 to 980 cells per ml water, accounting for 0.00057-0.0098% of the total bacteria encountered with beach water contact. These results illustrate that resistance to antibiotics by *Vibrio*- like bacteria is widespread on local recreational marine beaches. Although these resistant bacteria are a small percentage of the total bacteria, they may represent a potential public health issue through the introduction of resistance genes into human microbiomes during recreation or shellfish consumption.

INTRODUCTION

Antibiotics are among the most important discoveries of the 20th century and have revolutionized the way humans treat disease (1). However, their immense benefits come at the cost of increasing antibiotic resistance (AR), a natural phenomenon that is exacerbated by the extensive use of antibiotics for human (2, 3) and animal health (4) and for increasing yields in agricultural and aquaculture production (2, 5–7). Previous research has focused predominantly on AR in the clinical environment; but recently a holistic understanding called "One Health" has emerged in which human, animal, and environmental health are linked, and the entire system must be studied to understand the complex dynamics involved (8, 9). Pollution from anthropogenic sources such as hospitals (10–12), agriculture (13–18), human wastewater (19–24), and aquaculture (25–29) can contain not only antibiotics but both pathogenic and non-pathogenic bacteria carrying antibiotic resistance genes (ARG). When these wastes are released into the environment, native environmental bacteria that have little or no connection to human disease may be exposed to anthropogenic antibiotics or ARG. Environmental bacteria with naturally occurring resistance genes, or that have acquired genes, then serve as reservoirs for resistance, with the potential for the transfer back to pathogens and the emergence of resistant infections (30). The relative ease of gene transfer between bacteria is one of the primary reasons for interest in environmental antibiotic resistance (30). Despite a growing appreciation of these connections, studies of the environment as a reservoir of resistance have lagged behind research in clinical settings. The marine environment in particular is underexplored, even though humans routinely interact with the ocean for food and for recreation, providing an opportunity for antibiotic resistance transfer.

Vibrio species, a genus of Gram-negative bacteria, reside primarily in brackish and saltwater ecosystems, and include at least twelve pathogenic species including *V. cholera*, *V. alginolyticus*, and *V. parahaemolyticus* (31). *Vibrio* is of interest because most species are free living members of the marine bacterial community, while others are known for their symbioses with other marine organisms such as copepods (32), fish (33, 34), shellfish (31), and squid (35, 36). Previous research on AR has found antibiotic resistance in *Vibrio* species in a variety of marine areas around the world including the Baltic Sea (37, 38), the North Sea (38), Chesapeake Bay (39), South Carolina and Georgia (40, 41), Brazil (42), Peru (43), and India's Chennai coast (44). Percentage of resistance to at least one antibiotic varies from 8.3% (39) to 100% (45) which may be due to the site or to the variety of antibiotics tested in each study, but these results indicate a general prevalence in *Vibrio*. Prior studies have focused on one site, or multiple sites over a short period of time, preventing assessment of the persistence of antibiotic resistance and the effect of human activity and environmental conditions over time.

The purpose of this study was to assess the prevalence and persistence of antibiotic resistance in *Vibrio*-like bacteria at marine beaches with varying levels of human activity by conducting a temporal and spatial survey. The hypotheses were that beaches with greater human activity (denser urbanization, higher visitors) will have more isolates with antibiotic resistance and multiple resistance than secluded beaches, and that there will be a seasonal trend of more resistant bacteria in summer compared to winter and spring. The secluded beaches were selected to provide a baseline for the natural level of antibiotic resistant bacteria in the coastal marine environment. Finally, estimates of the amount of

resistant *Vibrio*-like bacteria encountered by humans through recreation or shellfish consumption were made to assess the potential for public health risk.

METHODS

Site Description

Six field sites were chosen near Falmouth, Massachusetts on Cape Cod (Figure 1). The sites represent different watersheds, bodies of water (Buzzards Bay (BB) vs. Waquoit Bay (WB)), levels of human activity, and salinity gradients (for the Waquoit Bay estuarine sites). The levels of human activity were assessed by the authors based on how frequently the beaches were used and how accessible they were. The site descriptions are given in detail in Table 1. A seventh site, Elizabeth Island, was sampled once in September 2015 to represent a more isolated site.

Field sampling

Samples of wet and dry sand and water were collected in June, July, August, September, October and December of 2014 and February, April and May of 2015. At each site, three one liter water samples were collected at ~45 cm depth using sterilized Nalgene bottles. Three sterile 50 ml centrifuge tubes of wet sediment were collected right above the water line on the beach, and three tubes of dry sediment were collected around the high tide line.

For environmental measurements, a YSI Professional Plus data sonde (Yellow Springs, OH) was used to measure barometric pressure, dissolved oxygen, water & air temperatures, specific conductivity, and salinity. Previous rainfall was based upon the rainfall amounts for the previous two days and the day of sampling obtained from Weather

Underground (wunderground.com) using the weather history custom tab. For Little Island (LI) site, the KMAFALM06 station was used. For Old Silver Beach (OSB) site, KMANORTH39 station was used. For Waquoit Bay sites (SCB, WB, NRB, BDN), the station KMAEASTF1 was used. Amount of sunlight minutes for each site was calculated using an online almanac http://www.almanac.com/astronomy/rise/zipcode/02540/) and looking up the sunrise time for each of the sampling days. The amount of sunlight was then determined by subtracting the sunrise time from the sampling time. This method was an estimate and did not include cloudiness as a factor. Each liter of water was subsampled and measured for turbidity using the MicroTPW detection system (HF Scientific) and the three measurements were averaged.

Sample Processing

Three replicate water samples for each site were subsampled and mixed to create one composite sample used to cultivate bacteria. The three replicate samples of each sand type (wet or dry) were combined and carefully mixed, and 10 gm was returned to a 50 ml centrifuge tube to create a composite sample to be used for culturing. 20 ml of sterile 1x phosphate buffered saline (PBS) was added to the 10 gm of sand, shaken for two and a half minutes and allowed to settle. This PBS mixture was then used for bacterial cultivation.

Cultivation of Vibrio bacteria

For samples between June 2014 and August 2014, alkaline peptone enrichment was used for the cultivation of *Vibrio* spp. A direct plating method was utilized for the samples collected after August 2014. For water and sediment samples collected between

June 2014 and August 2014, alkaline peptone enrichment was used prior to plating on Thiosulfate-Citrate-Bile-Sucrose (TCBS). Twenty five ml of water or 5 ml of the 1 X PBS elutant from sediment was inoculated into 225 ml of alkaline peptone media. All samples were incubated at 35-37ºC with shaking at 100 rpm for 6 hours. The incubated samples were then serially diluted with sterile seawater (direct inoculation or between $10^1 - 10^6$) and 100 µl was spread onto TCBS agar plates for cultivation of *Vibrio* species. TCBS plates were incubated at 35-37°C for 18-24 hours. Direct plating was accomplished for all subsequent samples by spreading 100 to 500 µl of the water sample or the PBS sediment mixture onto the TCBS agar and incubation at 35-37ºC for 18-24 hours.

Each plate total colony number was counted and up to three colonies of each morphology type on a given plate were picked. Picked colonies were grown in seawater broth and 800 µl of the culture was added to 200 µl of sterile 80% glycerol and stored at -80°C as sample stocks.

Antibiotic Resistance Testing

Antibiotics tested in this study were amoxicillin/ clavulanic acid, ciprofloxacin, doxycycline, oxytetracycline, and trimethoprim (Table 2). Glycerol stocks were used to inoculate seawater broth, and the cultures were incubated at 35-37°C for 8 hours. Cultures were adjusted to a 0.5 McFarland standard (OD_{600} of 0.15-0.20) and used to swab Muller Hinton plates for growth of lawns following the procedure by CLSI (46–48). Antibiotic discs were placed individually on each plate using sterilized forceps followed by incubation at 35-37°C for 16-18 hours. Inhibition zones around each disc were measured and recorded. Zone diameters were used to categorize sensitivity, intermediate

resistance, and resistance to the antibiotic. Resistance in this report includes both resistance and intermediate resistance, following previous literature (49). Breakpoints used for each antibiotic are shown in Table 2. Doxycycline and trimethoprim did not have breakpoints available for *Vibrio* spp., therefore breakpoints from Enterobacteriaceae were used.

16s ribosomal RNA gene Sequencing

A portion of the 16S ribosomal RNA gene was amplified using the primers 27F and 680R (*Vibrio*- specific primer, 49) and sequenced from isolates that were successfully tested for antibiotic resistance (406 total). Forty µl of cell culture was combined with 40 µl of 1X PBS in a PCR tube, lysed by incubation at 65ºC 30sec, 8ºC 30sec, 65°C 90sec, 97°C 180sec, 8°C 60sec, 65°C 180sec, 97°C 60sec, 65°C 60sec, and 80°C 10mins (pers comm Paul Kirchberger). These cell lysates were amplified using the 16S primers 27F and 680R (*Vibrio*- specific primer, 49). PCR products that showed a product of the correct size (650 base pairs) on a 1.5% agarose gel were purified using MinElute PCR Purification Kit (Qiagen, Hilden, Germany). Samples were sent to GeneWiz (South Plainfield, NJ, USA) for sequencing using primer 27F. Resulting chromatograms were assessed using 4Peaks (Amsterdam, Netherlands) and were corrected manually according to confidence in the chromatogram profile.

Final sequences (388 total) were submitted to BLAST and a best sequence identity was chosen based on percent identity and top hits. Unique sequences were assessed using mothur (51) and were then combined with Silva (v.1.2.11 online) *Vibrio* type sequences (search criteria: organism name- *Vibrio*, sequence quality- >90, strain: type).

Both the unique sequences and the type sequences were aligned with SINA aligner (52) available on Silva Online. Molecular phylogenies were constructed using Mega (53) with Partial Maximum Likelihood. Isolates that showed most similarity with *Vibrio*, *Photobacterium*, and unidentified organisms within the *Vibrio* genus were retained in the study, whereas isolates similar to *Aeromonas* (1), *Bacillus* (1), *Exiguobacterium* (14), *Oceanimonas* (3), *Oceanisphaera* (1), *Shewanella* (7), *Staphylococcus* (1), and samples unable to be sequenced (18) were eliminated. The *Vibrio*/*Photobacterium*/unidentified sequences were clustered into OTU groups based at 99% using mothur (51). Sequences have been deposited in GenBank (54).

arcGIS

Spatial analysis of these sites was accomplished to have a quantitative measurement of the degree of human impact at each sampling site. Spatial analysis was completed using arcGIS (ArcMap 10.4, run in Virtual Box on a Mac, Esri, Redlands, CA) in order to acquire land use information and population density around the field sites. Both watershed and proximity were evaluated/examined. Proximity analysis extended to a radius of 840 meters around each site (Figure 1). Watershed analysis compared sites using the embayments (also known as subwatersheds) on Cape Cod, available via the arcGIS online tool (search for embayments) or via Open Data (55). For all analyses, census, long-term care facility, hospital data, land use, and impervious surface were utilized. Information on downloading data and models are available for download (56).

Estimations of amounts of antibiotic resistant bacteria present in water

Using the September 2014, for all six regularly sampled sites, and September 2015, for the Elizabeth Island site, water samples, the total number of *Vibrio*-like bacteria recovered from each sample was estimated from petri dish counts, divided by the amount of inoculum in milliliters. The percentage of total *Vibrio* isolates resistant to 0, 1, 2, 3, or 4 antibiotics was then calculated by dividing the number of resistant isolates by the total for each sample. This percentage was multiplied by the estimated total of *Vibrio*-like bacteria recovered from each sample to determine the number of cells in a particular category that would be encountered in a milliliter of water. The number of cells resistant to at least one antibiotic to provide the total number of resistant cells per ml. These estimates were multiplied with ingestion estimates of how many milliliters of water individuals ingest in various recreational activities to estimate human exposure during beach recreation (57). Shellfish filtration estimates were produced by using published ranges of filtration for quahogs (*Mercenaria mercenaria*) and soft clams (*Mya arenaria*) (58). For the Eastern oysters (*Crassostrea virginica*), the filtration rate of 1.5 to 10 L/h/g dry weight (59) was multiplied by the average dry tissue size of Cape Cod oysters, with a range from 1.36 to 2.7 g dry tissue weight (60) to produce a range of filtration of 2.04 – 4.05 L/h. These filtration estimates, both low and high range, were multiplied by the number of resistant cells to estimate how many culturable resistant cells a shellfish might filter in one hour.

Multiple antibiotic resistance

The Multiple Antibiotic Resistance (MAR) index was calculated for each individual sample taken at a specific time and location, as previously described (61). A single MAR index was calculated for all samples to be indicative of the larger beach community.

Statistical analysis

For statistical analyses, Primer 6 and PERMANOVA were used (Auckland, New Zealand) (63, 64). The antibiotic resistance data was organized into two different matrices in order to examine patterns in the data. The first matrix was used to examine antibiotic resistance patterns on an isolate basis, and consisted of each bacterial isolate as a sample and the sensitivity/resistance to each antibiotic as the variable. The second matrix was used to examine resistance to multiple antibiotics and consisted of each location/sample time with the percentage of isolates resistant to 0, 1, 2, 3, or 4 antibiotics as the variables.

Permutational multivariate analysis of variance (PERMANOVA, Primer v. 6) was used to test two questions: whether human impact affects resistance patterns or prevalence, and whether watershed affects resistance patterns or prevalence (63). The isolate matrix was used to answer the question of resistance patterns, and the resistance to multiple antibiotic matrix was used to examine resistance prevalence. PERMANOVA tests examined a Bray-Curtis resemblance matrix (with a dummy variable to eliminate zeros) from the AR data compared to factors associated with the data. Tables 3 and 4 show the PERMANOVA tests and run parameters including the Sum of Squares- Type III (partial) and the permutation as unrestricted permutation of raw data. If p-values were greater than 0.25 and had a negative variation component, the factor was pooled.

For OTU-based community composition data, the matrix included each sample location/ month (for example: LI August) as the sample and the counts of OTU groups present as the variables. PERMANOVA tests were done using the same set-up and analysis as for the antibiotic resistance data, though here, the data were transformed using a log(x+1), and no dummy variables were used in creating the resemblance matrix.

To assess if environmental and land use variables were significant in structuring antibiotic resistance and community composition patterns, distance-based linear models (DistLM) in PERMANOVA+ were utilized using the Best procedure that examines all possible combinations along with the Bayesian Information Criterion (BIC) that provides a penalty for extraneous predictor variables (63). Environmental variables included amount of sunlight (minutes), average turbidity (NTU), barometric pressure (mmHg), salinity (ppt), dissolved oxygen (mg/L), water temperature (degrees Celsius), air temperature (degrees Celsius), and previous rainfall (inches).

Two separate land use variable spreadsheets were created: one from the Watershed Model (for the three watersheds- West Falmouth Harbor, Waquoit Bay, and Falmouth Old Silver Beach), and the other for the Proximity Model for each of the six regularly sampled sites (LI, OSB, SCB, WB, NRB, BDN). Each matrix included percentage vacant housing, total population, the mean of the median age, percentage of males in the population, percentage of population 5 years old and under, percentage of population 6 to 18 years old, percentage of population greater than 65 years old, percentage of impervious surfaces, and percentage urbanized land use. Land use categories that are considered urbanized were: mining, multi-family residential, high density residential, medium density residential, low density residential, commercial, industrial, transitional, transportation, waste disposal, powerline/utility, golf course, urban public/ institutional, cemetery, very low density residential, and junkyard. It should be noted that none of the analyses had long term care facilities or hospitals in these areas.

RESULTS

Antibiotic resistance of Vibrio spp. isolates

A total of 550 bacterial isolates were collected from beaches. Throughout the process, isolates were eliminated because they did not re-grow from glycerol stocks, were not able to be tested for disk diffusion or sequenced, or were not *Vibrio*-like bacteria. 360 of the bacterial isolates were *Vibrio*-like based upon sequence data and successfully tested using disk diffusion, and are reported on here. The majority of the tested isolates were resistant to at least one antibiotic (73.1%, 263 isolates) (Figure 2, Panel A), and 32.5% (117 isolates) were resistant to more than one antibiotic. There are more isolates with resistance to multiple antibiotics than there are isolates sensitive to all antibiotics. Few isolates were resistant to four antibiotics (5 isolates, 1.38%), and no isolates were resistant to all five of the tested antibiotics. Resistance varied based on antibiotic tested (Figure 2, Panel B): more resistance was observed to trimethoprim (47.5%), ciprofloxacin (30%), and amoxicillin (29.4%) than to oxytetracycline (5.8%) and doxycycline (4.2%). Resistance was present throughout the year and varied seasonally (Figure 2, Panel C). Spring, summer, and fall had higher percentages of isolates resistant to at least one antibiotic compared to the winter, though there were also fewer isolates in the winter. Based upon sample type, 71.5% of dry sand (98/137), 76.2% of wet sand (109/143), and 70% of water isolates (56/80), were resistant to at least one antibiotic (data not shown). The most prevalent antibiotic resistant categories were trimethoprim (28.9%), trimethoprim/ ciprofloxacin (18.3%), amoxicillin (16.3%), ciprofloxacin (9.9%), trimethoprim/amoxicillin/ciprofloxacin (7.6%), and trimethoprim/amoxicillin (6.6%) (Figure 2, Panel D).

Bacterial isolates were recovered over the entire study time (Figure 3), though reduced recovery occurred during the winter (Table 3). As indicated in Figure 3, some samples had less than or equal to 5 isolates. The proportion of isolates resistant to at least one antibiotic was variable between sites and within a given site over the sampled months (Figure 3). The Elizabeth Islands (EI) site, which was sampled as a very low impact control once on September 2015, had 20/24 isolates (83.3%) resistant to at least one antibiotic. At only three sample locations and time points (SCB 2014-06, BDN 2015-02, and WB 2015-02) were no resistant isolates present. Further, these samples also had very few isolates recovered (SCB 2014-06 and BDN 2015-02 with 1 isolate each and WB 2015-02 with 2 isolates).

MAR index results showed variability between 0 - 0.538 throughout the samples by location and time (Figure 4), and EI's MAR value for September 2015 was 0.225. 47.6% of the samples have a MAR value of equal to or greater than 0.2. A collective MAR value for *Vibrio*-like bacterial isolates at all of the beaches was 0.233.

Statistical analyses

The isolate matrix was used in two PERMANOVA tests to determine if human impact and/or watershed affect resistance patterns. For both human impact and watershed, the only factor with significance was the interaction of location and month, explaining 12.2% of the human impact related variance and 11% of the watershed related variance (Table 4). The multiple antibiotic resistance matrix allowed examination of human impact and watershed impact on the prevalence of multiple resistance. For both tests, month alone was significant and explained about 9% of the variation (Table 4). In

the watershed test, the interaction of watershed and month was also significant, explaining 10% of the variation. In all cases, the residual variance was ≥ 15%.

DistLM was used to assess the effect of environmental variables (sunlight time, turbidity, barometric pressure, salinity, dissolved oxygen, water temperature, air temperature, and previous rainfall) in explaining the isolate antibiotic resistance variation. The best BIC model included sunlight time, barometric pressure, and previous rainfall, with a R^2 value of 0.12935 (12.9%). The isolate matrix was also compared to watershed and proximity land use variables. The watershed best BIC model included impervious land use with a R^2 of 2.799×10^{-2} (2.8%), while the proximity best BIC model was the factor of percentage of population ages 6 to 18 with a R^2 of 2.2509×10^{-2} (2.3%). When the multiple antibiotic resistance matrix was compared to environmental variables using the DistLM test, the best BIC test was a model with sunlight time and dissolved oxygen with a R^2 value of 0.17034 (17.0%). The multiple resistance matrix was also compared to the proximity and watershed variables and both tests indicated percentage of population male as the top factor (R^2 of 5.2889×10^{-2} (5.3%) and 5.3429×10^{-2} (5.3%) respectively).

Community composition of Vibrio isolates

The *Vibrio*-like bacterial diversity changes over time (Figure 5), with some OTUs abundant and present throughout the year while others are less abundant and seen at specific points. Individual OTUs can harbor different resistance patterns, even for OTU groups that are not dominant (Figure 6). OTU group 17 and OTU group 8 represent the most abundant isolates (Figure 5). They not only contain diverse resistant patterns throughout the year, they each have a different subset of abundant resistance types (Figure

5). The isolates in OTU 8 show most resistance to amoxicillin and multiple antibiotic resistance to amoxicillin/ciprofloxacin, and oxytetracycline,/doxycycline/amoxicillin (Figure 7, Panel A) (56). For OTU 17, isolates show most resistance to trimethoprim and ciprofloxacin and have multiple resistance to trimethoprim/ciprofloxacin, trimethoprim/amoxicillin/ciprofloxacin and trimethoprim/amoxicillin (Figure 7, Panel B) (56).

PERMANOVA tests examining OTU composition compared to human impact indicate that month was the only significant factor. Month remained significant even when other factors were pooled (Table 4). A PERMANOVA test examining watershed importance in OTU composition also indicated month as the only significant factor even after pooling. A DistLM Best BIC test analyzing OTU composition with environmental variables showed the best explanatory variable was previous rainfall with an R^2 of 8.1966 x 10^{-2} (8.2%). In examining OTU composition to both proximity and watershed land use models, mean median age illustrates an R^2 of 2.2174 x 10^{-2} (2.2%) and impervious surfaces represents an R^2 of 2.8351 x 10^{-2} (2.8%), respectively.

Estimations of resistant Vibrio-like bacteria in marine recreation and shellfish filtration

The amount of resistant bacteria present was estimated and used to project the amount encountered by humans engaging in recreational activities (Table 6). Human exposure varies primarily with the amount of water ingested during a particular activity. Children's swimming has the highest amount ingested (37 milliliters of water), and results in an estimated 2,109- 36,260 resistant bacteria ingested, depending on site. Rowing has

the lowest value at 3.5 ml, and an estimated 199.5 to 3,430 resistant bacteria ingested, depending on site.

Estimates of how many resistant bacteria a shellfish might filter in an hour were calculated for *Crassostrea virginica* (Eastern oyster), *Mercenaria mercenaria* (quahog/hard clam), and *Mya arenaria* (soft clams) (Table 7). *M. mercenaria* has the lowest estimates for filtration with 0.3-3.6 liters filtered per hour, while *C. virginica* has the most with 2.04-27 liters per hour. *C. virginica* is estimated to filter between $1.2 \times 10^5 – 2.6 \times 10^7$ resistant cells per hour. *M. mercenaria* is estimated to filter $1.7 \times 10^4 – 3.5 \times 10^6$ resistant cells per hour and *M.arenaria* filters $9.7 \times 10^4 – 7.2 \times 10^6$ resistant cells per hour.

DISCUSSION

This study was undertaken to examine the amount of antibiotic resistance present in *Vibrio*-like bacteria isolated from marine recreational beaches over temporal and spatial scales and with a gradient of human impact. Antibiotic resistance was found to be widespread in the studied samples with 73% resistant to at least one antibiotic and 32.5% resistant to multiple antibiotics. Overall, these reported values are consistent with previous research findings, and demonstrate that resistance to human-used antibiotics is not an anomaly for *Vibrio*-like species on Cape Cod. Studies examining *Vibrio* isolates from water and sediment in a Brazilian shrimp farm (45), and seawater samples from Peru (41), showed all isolates resistant to at least one antibiotic. *Vibrio* isolates from sediment and water from three sites on India's Chennai coast showed ranges from 70% to 85% resistance to at least one antibiotic, depending on sample type and location (44). Studies have also looked at specific pathogenic *Vibrio* species to determine their sensitivity to

antibiotics. In samples taken from recreational and commercial regions of the Chesapeake Bay, about 86.2% of *V. vulnificus* and 91.2% of *V. parahaemolyticus* expressed intermediate resistance to at least one antibiotic (39). 19.3% of *resistant V. vulnificus*. and 70.6% *V. parahaemolyticus* were categorized as having expressed resistance to at least one antibiotic (39). At two industrially contaminated sites and an uncontaminated control site in South Carolina and Georgia, 99.3% (150/151) of *V. vulnificus* isolates exhibited resistance to at least one antibiotic (40); and at these same sites, about 99.4% (348/350) *V. parahaemolyticus* isolates were resistant to at least one antibiotic (41).

Resistance in Vibrio isolates varies by antibiotic.

The fraction of isolates resistant to individual antibiotics in our study was 47.5% for trimethoprim, 30% for ciprofloxacin, 29.4% for amoxicillin, 5.8% for oxytetracycline and 4.2% for doxycycline. Resistance prevalence results by antibiotic from other published studies are quite variable. Trimethoprim resistance ranges from 0% to less than 5% (38) and up to 72.5% (64), while ciprofloxacin resistance has values of 0% (39, 40, 45, 64) to 22-36% (37). Amoxicillin resistance values from other marine studies range from 0% (39) to < 5% (38) to 56-81% (37). Oxytetracycline has shown resistance from <3% (40), 10-32% (37), 10.8-12.1% (45), and 99.4% (64). From one study, marine bacterial isolates exhibited no resistance to doxycycline (39). This variability may be due to locational differences in species composition of *Vibrio* or to the varied ways of reporting data. Some studies report resistance as a breakpoint, while others report resistance and intermediate breakpoints together, as this study does. In this study, the differences in resistance could

be due to a variety of factors including variations in structural targets (Table 2) and the role of resistance in the genus *Vibrio*.

Multiple antibiotic resistance

The MAR index of almost half of the samples in this study (47.6%) was greater than or equal to 0.2. In Krumperman's study on fecal contamination of food, samples from more "natural" areas (orchards, domesticated animals, wild animals) had MAR indices of less than 0.2, while samples from more anthropogenically affected areas (sewage, brooder houses, piggeries) had MAR index values of between 0.312-0.630 (61). Therefore, an index value of greater than 0.2 was suggested to represent areas impacted by point-source contamination. A study of antibiotic resistance in coastal vertebrates, including seabirds and seals, showed MAR values of greater than 0.2 for 38% of resistant bacterial isolates (65). The sites in this study are not samples with point source contamination, yet have higher values on Krumperman's Index. This finding may indicate that Krumperman's index is not representative of impact for naturally affected areas due to its underlying assumptions about multiple resistance as prevalent for anthropogenically affected areas. Further, Krumperman's index does not take into account the mechanism of resistance, which is discussed in more detail below.

Community composition of Vibrio isolates illustrates temporal variation and shows multiple resistances in a given OTU.

Vibrio-like isolates were recovered throughout the entire year on Cape Cod and therefore are present throughout the year in cultivatable forms. Winter had reduced recovery, which is expected due to the seasonal cycling of vibrios (50, 66) and community structure changed over time and showed varying OTUs as dominant (50). In examining

OTU composition relative to human impact and watershed using PERMANOVA (Table 5), month was the only significant factor for both tests, which supports the strong seasonality of *Vibrio*. DistLM results indicated previous rainfall as the variable best fitting the OTU composition data, which relates to increased runoff and reduced salinity in the nearshore marine environment.

A majority of the non-singleton OTUs showed resistance to multiple different antibiotics. This result indicates that specific resistances are not likely associated with specific strains, and that there may naturally be a diverse selection of antibiotic resistance in the marine environment. OTU 8 and 17 were the most prevalent bacterial groups and had differing types of resistance. The resistance differences may be due to the mechanisms of how these resistances are carried, or these may be due to species/strain-level variation.

While sequences were too short to reliably assign taxonomic affiliations at the species level, OTU groups 6, 17 and 19 showed similarity to *V. alginolyticus* and *V. parahaemolyticus*, which is consistent with prior studies reporting antibiotic resistance in pathogenic *Vibrio* species from the marine environment (37, 38). With this in mind, it is important to consider antibiotic resistance in the context of climate change. A previous study has documented increasing *Vibrio* abundance and infections with increasing sea surface temperatures (67). With the prevalence of resistance in *Vibrio* shown in this study, and the projected increases in *Vibrio* abundance, the likelihood of encountering an antibiotic resistant *Vibrio* bacteria and contracting an infection could increase with a warming climate, though further research is necessary to elucidate the extent.

Human impact, watershed, land use and environmental factors have limited effect on antibiotic resistance.

PERMANOVA tests to assess if human impact or watershed affected resistance patterns indicated that neither exerted a strong influence. The only significant factor was the interaction effect of location and month, which explained less than 12% of the variation in each test. Tests examining the same questions for multiple resistance prevalence also showed month and the interaction of watershed and month as significant. This result is likely based on changes in bacterial species composition over time, which impacts the types of resistance present. Lack of significance to individual factors of human impact and watershed suggest that these were not significantly different at each of the sites. The residual variation was still fairly high for all of the tests, indicating that there are likely other factors that influence the resistance patterns that have not been accounted for in this work.

The environmental variables of sunlight time, barometric pressure, dissolved oxygen and previous rainfall best explained the variation in antibiotic resistance patterns or multiple antibiotic resistance amounts in DistLM analysis. These environmental variables also likely have to do with the bacterial community composition. Previous rainfall could influence the salinity of the water and/or contribute to the release of environmental bacteria from sand and impervious surfaces, thereby changing which OTUs may more readily thrive in the area, and ultimately what resistance may be most prevalent. Sunlight time is also indicative of seasonality for this area, which also ties into community composition changes.

The land use variables impervious surfaces and percentage of the population ages 6 to 18 years old explained less than 3% of the variability in the data in DistLM tests. This may suggest that on a proximity level, population of the community around the beach may be more important, whereas land use is of greater impact on a watershed level. The human population structure may affect resistance by affecting what antibiotics are used in the proximal areas, though it seems unlikely that a direct relationship exists. Impervious land use could affect runoff that impacts particular beaches and could influence OTU composition, thereby influencing what types of resistance are present. Land use information was obtained from the 2010 Census data; and land use was not directly measured during the sampling period of 2014 to 2015 for this study. Perhaps part of the lack of specificity to particular land use variables is due to this temporal inaccuracy.

Overall, lack of specific relationships to particular land use variables, along with no significant relationships of antibiotic resistance data to individual factors such as watershed or human impact, and the pervasiveness of resistance throughout the study, indicate that these sites are all similar in environmental and human impact, at least considering the variables that were measured. This result is further strengthened by results from the isolated site on the Elizabeth Islands. Though this site was only sampled once, in September 2015, 83.3% of samples were resistant to at least one antibiotic, despite generally limited human access and activity at the site. Another aspect to consider is that the coastal ocean may be permeated with antibiotic exposure over time, with the mixture of natural and anthropogenic use of antibiotics. With the coastal ocean considered replete with antibiotic exposure, there would be no differences between impact levels. It

is also possible that conventional human impact indicators are not relevant on the microscale that bacteria experience. The antibiotic resistance research community may want to consider selecting human activity indicators with this in mind.

Estimates of antibiotic resistant bacteria indicate potential for human interaction through recreation and shellfish.

Humans routinely interact with the ocean through recreation and food consumption. If antibiotic resistant bacteria are present, it is possible that humans could contract a resistant infection, perhaps directly from a pathogenic *Vibrio*. Alternatively, they could ingest a resistant *Vibrio* that could transfer its resistance gene to another microbe in the person's GI tract or on their skin, therefore increasing resistance potential in the future. The estimates shown in this study indicate that antibiotic resistant culturable *Vibrio* are a small proportion of the total bacteria ingested via beach water (0.00057-0.0098 %). Although small, the real impacts are unknown. One could imagine that despite the potential for frequent occurrences suggested by the widespread and persistent presence of resistance, if the transfer rate is extremely low, the potential risk of a resistant infection would be small. For a back of the envelope calculation, if 57-980 resistant, culturable *Vibrio*-like bacteria are found in a milliliter of water (based on these estimates by site) and 10% have the ability to transfer, that leaves 5.7-98 resistant, culturable, *Vibrio*-like transferable bacteria per milliliter of water. Although occurrences and potential for harm seem small but possible, further research is necessary to determine actual risk. A key question is whether interactions with resistant bacteria result in an increased likelihood of gaining a resistant infection. Answering this question requires understanding the mechanism of resistance and its ability to be transferred. Knowledge of what resistance

genes are common in the environment, their importance in health clinics, along with transfer rates of resistance from marine bacteria to bacteria present on the human skin or in the GI tract would be useful to quantify the rate at which resistance genes are likely to be shared.

To put this work into context, it may also be helpful to study amounts of antibiotic resistance in other environments that humans routinely interact with. Resistance levels in the ocean may not present greater risk than those in other areas that humans encounter such as built surfaces such as bathrooms, kitchens, and desks, or even own body parts such as hands.

High levels of resistance in Vibrio may be indicative of the ecological role of antibiotics and antibiotic resistance beyond competition for resources.

The reported prevalence of antibiotic resistance in local *Vibrio* bacteria may be due to the bacteria's ability to produce and resist antibiotics. A study examining pelagic marine bacteria illustrated that gamma proteobacteria of the orders Alteromonadales and Vibrionales (which includes the genus *Vibrio*; 74), readily produced inhibitory agents and were resistant to such molecules (69). Further research into *Vibrionaceae*, a family that includes *Vibrio* and *Photobacterium* (68), showed this group readily exhibited resistance to antibiotics, although only some members produce antibiotics (70). It is likely that antibiotics have a diverse set of uses in nature beyond growth inhibition, including signaling, especially at the lower concentrations that would normally be seen in the environment (71). To this end, it is thought that some antibiotic resistances may originally have had different purposes; MDR efflux pumps that allow for resistance to quinolones may have allowed for signaling, and the beta lactamases may have originally worked to

make peptidoglycan (71). Even further, intrinsic resistances may have been a part of bacteria living in diverse regions such as rhizospheres, where they would encounter a variety of toxic compounds (71). What is now categorized as antibiotic resistance, especially in environmental settings, likely has different evolutionary purposes other than the clinical use of antibiotics.

One specific type of intrinsic resistance is efflux pumps, which transport materials out of cells (including antibiotics; 69), and can be prevalent in Gram negative bacteria (72). Gram negative bacteria are prevalent in the ocean (73) and *Vibrio* are one example of these bacteria. Although the mechanism of resistance was not tested, it seems possible that efflux pumps may represent a mechanism for the high rate of resistance seen in this study. A previous functional metagenomic study on antibiotic resistance found that the majority of known antibiotic resistance genes prevalent in ocean samples were multidrug efflux pumps (74). It is possible that the high amounts of resistance seen in the environment may not necessarily be due to the presence of specific antibiotic resistance genes, but to the evolutionary importance of being able to pump materials out of a cell.

CONCLUSION

This study illustrates that antibiotic resistance can be prevalent in *Vibrio*-like bacteria present at low human impact marine beaches. Although bacterial prevalence is related to seasonal conditions, resistance persists despite temporal, spatial, and environmental differences. Community structure of *Vibrio*-like bacteria varied over time, with two sequence-based taxonomic groups most dominant. Resistance to a specific antibiotic was not associated with a particular group, but the two most abundant groups did show

resistance to different subsets of antibiotics. Ingestion and filtration estimates indicate that future research focusing on transfer of resistance from environmental bacteria would be useful for estimating potential risk to public health. In addition, future studies should examine the mechanisms of antibiotic resistance present in the marine environment and what other ecological and competitive benefits the resistance genes provide to improve insight into the origin and importance of resistance evolutionarily.

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REFERENCES

1. **Davies, J, & Davies, D.**, **Davies J**, **Davies D**. 2010. Origins and Evolution of Antibiotic Resistance. Microbiol. Mol. Biol. Rev. **74**:417–433.
2. **Martinez JL**. 2009. Environmental pollution by antibiotics and by antibiotic resistance determinants. Environ. Pollut. **157**:2893–2902.
3. **Alanis AJ**. Resistance to antibiotics: are we in the post-antibiotic era? Arch. Med. Res. **36**:697–705.
4. **Sarmah AK**, **Meyer MT**, **Boxall ABA**. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere **65**:725–59.
5. **Kümmerer K**. 2009. Antibiotics in the aquatic environment - A review - Part I. Chemosphere **75**:417–434.
6. **Allen HK**, **Donato J**, **Wang HH**, **Cloud-Hansen K a**, **Davies J**, **Handelsman J**. 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. **8**:251–9.
7. **Kümmerer K**. 2004. Resistance in the environment. J. Antimicrob. Chemother. **54**:311–20.
8. **Finley RL**, **Collignon P**, **Larsson DGJ**, **Mcewen SA**, **Li XZ**, **Gaze WH**, **Reid-Smith R**, **Timinouni M**, **Graham DW**, **Topp E**. 2013. The scourge of antibiotic resistance: The important role of the environment. Clin. Infect. Dis. **57**:704–710.
9. **Robinson TP**, **Bu DP**, **Carrique-Mas J**, **Fèvre EM**, **Gilbert M**, **Grace D**, **Hay SI**, **Jiwakanon J**, **Kakkar M**, **Kariuki S**, **Laxminarayan R**, **Lubroth J**, **Magnusson U**, **Thi Ngoc P**, **Van Boeckel TP**, **Woolhouse MEJ**. 2016. Antibiotic resistance is the quintessential One Health issue. Trans. R. Soc. Trop. Med. Hyg. **110**:377–380.
10. **Jakobsen L**, **Sandvang D**, **Hansen LH**, **Bagger-Skjøt L**, **Westh H**, **Jørgensen C**, **Hansen DS**, **Pedersen BM**, **Monnet DL**, **Frimodt-Møller N**, **Sørensen SJ**, **Hammerum AM**. 2008. Characterisation, dissemination and persistence of gentamicin resistant *Escherichia coli* from a Danish university hospital to the waste water environment. Environ. Int. **34**:108–15.
11. **Chang X**, **Meyer MT**, **Liu X**, **Zhao Q**, **Chen H**, **Chen J**, **Qiu Z**, **Yang L**, **Cao J**, **Shu W**. 2010. Determination of antibiotics in sewage from hospitals, nursery and slaughter house, wastewater treatment plant and source water in Chongqing region of Three Gorge Reservoir in China. Environ. Pollut. **158**:1444–50.
12. **Thompson JM**, **Gündoğdu A**, **Stratton HM**, **Katouli M**. 2013. Antibiotic resistant *Staphylococcus aureus* in hospital wastewaters and sewage treatment plants with special reference to methicillin-resistant *Staphylococcus aureus* (MRSA). J. Appl. Microbiol. **114**:44–54.
13. **Silbergeld EK**, **Graham J**, **Price LB**. 2008. Industrial food animal production, antimicrobial resistance, and human health. Annu. Rev. Public Health **29**:151–69.
14. **Li L**, **Sun J**, **Liu B**, **Zhao D**, **Ma J**, **Deng H**, **Li X**, **Hu F**, **Liao X**, **Liu Y**. 2013. Quantification of lincomycin resistance genes associated with lincomycin residues in waters and soils adjacent to representative swine farms in China. Front. Microbiol. **4**:1–9.

15. **Chee-Sanford JC**, **Mackie RI**, **Koike S**, **Krapac IG**, **Lin Y-F**, **Yannarell AC**, **Maxwell S**, **Aminov RI**. 2009. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. J. Environ. Qual. **38**:1086–108.
16. **Ghosh S**, **LaPara TM**. 2007. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J. **1**:191–203.
17. **Sapkota AR**, **Hulet RM**, **Zhang G**, **McDermott P**, **Kinney EL**, **Schwab KJ**. 2011. Lower prevalence of antibiotic-resistance Enterococci on U.S. conventional poultry farms that transitioned to organic practices. Environ. Health Perspect. **119**:1622–1629.
18. **McEachran AD**, **Blackwell BR**, **Hanson JD**, **Wooten KJ**, **Mayer GD**, **Cox SB**, **Smith PN**. 2015. Antibiotics, Bacteria, and Antibiotic Resistance Genes: Aerial Transport from Cattle Feed Yards via Particulate Matter. Environ. Health Perspect. **123**:337–343.
19. **Bessa L**, **Barbosa-Vasconcelos A**, **Mendes Â**, **Vaz-Pires P**, **Martins da Costa P**. 2014. High prevalence of multidrug-resistant *Escherichia coli* and *Enterococcus spp*. in river water, upstream and downstream of a wastewater treatment plant. J. Water Health 426–436.
20. **Amos GCA**, **Zhang L**, **Hawkey PM**, **Gaze WH**, **Wellington EM**. 2014. Functional metagenomic analysis reveals rivers are a reservoir for diverse antibiotic resistance genes. Vet. Microbiol. **171**:441–447.
21. **Marti E**, **Jofre J**, **Balcazar JL**. 2013. Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. PLoS One **8**:1–8.
22. **Novo A**, **André S**, **Viana P**, **Nunes OC**, **Manaia CM**. 2013. Antibiotic resistance, antimicrobial residues and bacterial community composition in urban wastewater. Water Res. **47**:1875–87.
23. **Bouki C**, **Venieri D**, **Diamadopoulos E**. 2013. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: A review. Ecotoxicol. Environ. Saf. **91**:1–9.
24. **Rizzo L**, **Manaia C**, **Merlin C**, **Schwartz T**, **Dagot C**, **Ploy MC**, **Michael I**, **Fatta-Kassinos D**. 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. **447**:345–360.
25. **Labella A**, **Gennari M**, **Ghidini V**, **Trento I**, **Manfrin A**, **Borrego JJ**, **Lleo MM**. 2013. High incidence of antibiotic multi-resistant bacteria in coastal areas dedicated to fish farming. Mar. Pollut. Bull. **70**:197–203.
26. **Di Cesare A**, **Luna GM**, **Vignaroli C**, **Pasquaroli S**, **Tota S**, **Paroncini P**, **Biavasco F**. 2013. Aquaculture can promote the presence and spread of antibiotic-resistant *Enterococci* in marine sediments. PLoS One **8**:1–8.
27. **Yang J**, **Wang C**, **Shu C**, **Liu L**, **Geng J**, **Hu S**, **Feng J**. 2013. Marine sediment bacteria harbor antibiotic resistance genes highly similar to those found in human pathogens. Microb. Ecol. **65**:975–981.
28. **Chelossi E**, **Vezzulli L**, **Milano A**, **Branzoni M**, **Fabiano M**, **Riccardi G**, **Banat IM**.

2003. Antibiotic resistance of benthic bacteria in fish-farm and control sediments of the Western Mediterranean. Aquaculture **219**:83–97.
29. **Buschmann AH**, **Tomova A**, **López A**, **Maldonado MA**, **Henríquez LA**, **Ivanova L**, **Moy F**, **Godfrey HP**, **Cabello FC**. 2012. Salmon aquaculture and antimicrobial resistance in the marine environment. PLoS One **7**:26–28.
30. **Ashbolt NJ**, **Amézquita A**, **Backhaus T**, **Borriello P**, **Brandt KK**. 2013. Human Health Risk Assessment (HHRA) for Environmental Development and Transfer of Antibiotic Resistance. Environ. Health Perspect. **121**:993–1002.
31. **Pruzzo C**, **Huq A**, **Colwell RR**, **Donelli G**. 2005. Pathogenic Vibrio Species in the Marine and Estuarine Environment, p. 217–252. *In* Belkin, S, Colwell, RR (eds.), Oceans and Health: Pathogens in the Marine Environment. Springer.
32. **Huq A**, **Small EB**, **West PA**, **Huq MI**, **Colwell RR**. 1983. Ecological Relationships Between *Vibrio cholerae* and Planktonic Crustacean Copepods **45**:275–283.
33. **Halpern M**, **Izhaki I**. 2017. Fish as hosts of *Vibrio cholerae*. Front. Microbiol. **8**:1–7.
34. **Senderovich Y**, **Izhaki I**, **Halpern M**. 2010. Fish as reservoirs and vectors of *Vibrio cholerae*. PLoS One **5**.
35. **Lee K**, **Ruby EG**. 1994. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl. Environ. Microbiol. **60**:1565–1571.
36. **Nyholm S V**, **Mcfall-Ngai MJ**. 2004. The winnowing : establishing the squid- *Vibrio* symbiosis. Nat. Rev. Microbiol. **2**:632–642.
37. **Mudryk ZJ**, **Kosiorek A**, **Perliński P**. 2013. In vitro antibiotic resistance of *Vibrio*-like organisms isolated from seawater and sand of marine recreation beach in the southern Baltic Sea. Hydrobiologia **702**:141–150.
38. **Bier N**, **Schwartz K**, **Guerra B**, **Strauch E**. 2015. Survey on antimicrobial resistance patterns in *Vibrio vulnificus* and *Vibrio cholerae* non-O1/non-O139 in Germany reveals carbapenemase-producing Vibrio cholerae in coastal waters. Front. Microbiol. **6**:1–11.
39. **Shaw KS**, **Rosenberg Goldstein RE**, **He X**, **Jacobs JM**, **Crump BC**, **Sapkota AR**. 2014. Antimicrobial susceptibility of *Vibrio vulnificus* and *Vibrio parahaemolyticus* recovered from recreational and commercial areas of Chesapeake Bay and Maryland Coastal Bays. PLoS One **9**:1–11.
40. **Baker-Austin C**, **McArthur J V**, **Lindell AH**, **Wright MS**, **Tuckfield RC**, **Gooch J**, **Warner L**, **Oliver J**, **Stepanauskas R**. 2009. Multi-site analysis reveals widespread antibiotic resistance in the marine pathogen *Vibrio vulnificus*. Microb. Ecol. **57**:151–9.
41. **Baker-Austin C**, **McArthur JV**, **Tuckfield RC**, **Najarro M**, **Lindell AH**, **Gooch J**, **Stepanauskas R**. 2008. Antibiotic resistance in the shellfish pathogen *Vibrio parahaemolyticus* isolated from the coastal water and sediment of Georgia and South Carolina, USA. J. Food Prot. **71**:2552–2558.
42. **Paim I**, **Souza C De**, **Alice M**, **Saraiva F**. 2018. Antimicrobial resistance and potential virulence of *Vibrio parahaemolyticus* isolated from water and bivalve mollusks from Bahia, Brazil. Mar. Pollut. Bull. **131**:757–762.
43. **Sulca MA**, **Orozco R**, **Alvarado DE**. 2018. Antimicrobial resistance not related to 1,2,3 integrons and superintegron in Vibrio spp. isolated from seawater sample of

Lima (Peru). Mar. Pollut. Bull. **131**:370–377.
44. **Vignesh S**, **Muthukumar K**, **James RA**, **Arthur James R**. 2012. Antibiotic resistant pathogens versus human impacts: a study from three eco-regions of the Chennai coast, southern India. Mar. Pollut. Bull. **64**:790–800.
45. **Rocha RDS**, **Sousa OV de**, **Vieira RHS dos F**. 2016. Multidrug-resistant *Vibrio* associated with an estuary affected by shrimp farming in Northeastern Brazil. Mar. Pollut. Bull. 1–4.
46. **Clinical and Laboratory Standards Institute**. 2014. M100-S24: Performance Standards for Antimicrobial Susceptibility Testing, 24th ed. Wayne, PA.
47. **Clinical and Laboratory Standards Institute**. 2012. Performance Standards for Antimicrobial Disk Susceptibility Tests, 11th ed. Wayne, PA.
48. **Clinical and Laboratory Standards Institute**. 2006. M45-P: Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria ; Proposed Guideline. Wayne, PA.
49. **Alm EW**, **Zimbler D**, **Callahan E**, **Plomaritis E**. 2014. Patterns and persistence of antibiotic resistance in faecal indicator bacteria from freshwater recreational beaches. J. Appl. Microbiol. **117**:273–85.
50. **Thompson JR**, **Randa MA**, **Marcelino LA**, **Tomita-Mitchell A**, **Lim E**, **Polz MF**. 2004. Diversity and dynamics of a North Atlantic coastal *Vibrio* community. Appl Env. Microbiol **70**:4103–4110.
51. **Schloss PD**, **Westcott SL**, **Ryabin T**, **Hall JR**, **Hartmann M**, **Hollister EB**, **Lesniewski RA**, **Oakley BB**, **Parks DH**, **Robinson CJ**, **Sahl JW**, **Stres B**, **Thallinger GG**, **Horn DJ Van**, **Weber CF**. 2009. Introducing mothur : open-source, platform-independent , community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. **75**:7537–7541.
52. **Klindworth A**, **Pruesse E**, **Schweer T**, **Peplies J**, **Quast C**, **Horn M**, **Glöckner FO**. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. **41**:1–11.
53. **Kumar S**, **Stecher G**, **Tamura K**. 2016. MEGA7 : Molecular Evolutionary Genetics Analysis Version 7 . 0 for Bigger Datasets Brief communication **33**:1870–1874.
54. **May MK**. 2018. Sequence data for Antibiotic resistance in *Vibrio*-like bacteria is common at marine beaches on Cape Cod, MA. GenBank.
55. **Cape Cod Commission**. 2014. Embayments. ArcGIS Hub.
56. **May MK**, **Gast RJ**. 2018. Supplemental Information: Antibiotic resistance in *Vibrio*-like bactera is common on Cape Cod, MA beaches. Woods Hole Open Access Serv. Woods Hole.
57. **Leonard AFC**, **Zhang L**, **Balfour AJ**, **Garside R**, **Gaze WH**. 2015. Human recreational exposure to antibiotic resistant bacteria in coastal bathing waters. Environ. Int. **82**:92–100.
58. **Du Clos KT**, **Jones IT**, **Carrier TJ**, **Brady DC**, **Jumars PA**. 2017. Model-assisted measurements of suspension-feeding flow velocities. J. Exp. Biol. **220**:2096–2107.
59. **National Oceanic and Atmospheric Association**. 2007. Status Review of the Eastern Oyster (*Crassostrea virginica*) Report to the National Marine Fisheries Service. East. Oyster Biol. Rev. 115.

60. **Reitsma J**, **Murphy DC**, **Archer AF**, **York RH**. 2017. Nitrogen extraction potential of wild and cultured bivalves harvested from nearshore waters of Cape Cod, USA. Mar. Pollut. Bull. **116**:175–181.
61. **Krumperman PH**. 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Appl. Environ. Microbiol. **46**:165–170.
62. **Clarke KR**. 1993. Non-parametric multivariate analyses of changes in community structure. Aust. J. Ecol. **18**:117–143.
63. **Anderson M.**, **Gorley RN**, **Clarke JK**. 2008. Permanova+ for Primer: guide for software and statistical methods. PRIMER-E Ltd., Plymouth, UK.
64. **Igbinosa EO**. 2016. Detection and antimicrobial resistance of *Vibrio* isolates in aquaculture environments: implications for public health. Microb. Drug Resist. **22**:238–245.
65. **Rose JM**, **Gast RJ**, **Bogomolni A**, **Ellis JC**, **Lentell BJ**, **Touhey K**, **Moore M**. 2009. Occurrence and patterns of antibiotic resistance in vertebrates off the Northeastern United States coast. FEMS Microbiol. Ecol. **67**:421–31.
66. **Thompson JR**, **Pacocha S**, **Pharino C**, **Klepac-Ceraj V**, **Hunt DE**, **Benoit J**, **Sarma-Rupavtarm R**, **Distel DL**, **Polz MF**. 2005. Genotypic diversity within a natural coastal bacterioplankton population. Science **307**:1311–1313.
67. **Vezzulli L**, **Grande C**, **Reid PC**, **Hélaouët P**, **Edwards M**, **Höfle MG**, **Brettar I**. 2016. Climate influence on *Vibrio* and associated human diseases during the past half-century in the coastal North Atlantic. Proc. Natl. Acad. Sci. **113**.
68. **Farmer III JJ**, **Janda JM**. 2005. *Vibrionaceae*, p. 1–7. *In* Bergey's Manual of Systematics of Archaea and Bacteria. John Wiley & Sons, Inc.
69. **Long RA**, **Azam F**. 2001. Antagonistic interactions among marine pelagic bacteria. Appl. Environ. Microbiol. **67**:4975.
70. **Cordero OX**, **Wildschutte H**, **Kirkup B**, **Proehl S**, **Ngo L**, **Hussain F**, **Le Roux F**, **Mincer T**, **Polz MF**. 2012. Ecological Populations of Bacteria Act as Socially Cohesive Units of Antibiotic Production and Resistance. Science (80-.). **337**:1228–1231.
71. **Martinez JL**. 2009. The role of natural environments in the evolution of resistance traits in pathogenic bacteria 2521–2530.
72. **Webber MA**, **Piddock LJ V**. 2003. The importance of efflux pumps in bacterial antibiotic resistance. J. Antimicrob. Chemother. **51**:9–11.
73. **Giovannoni S**, **Rappé M**. 2000. Evolution, Diversity, and Molecular Ecology of Marine Prokaryotes, p. 47–84. *In* Kirchman, DL (ed.), Microbial Ecology of the Oceans,. Wiley-Liss.
74. **Hatosy SM**, **Martiny AC**. 2015. The ocean as a global reservoir of antibiotic resistance genes. Appl. Environ. Microbiol. **81**:7593–7599.

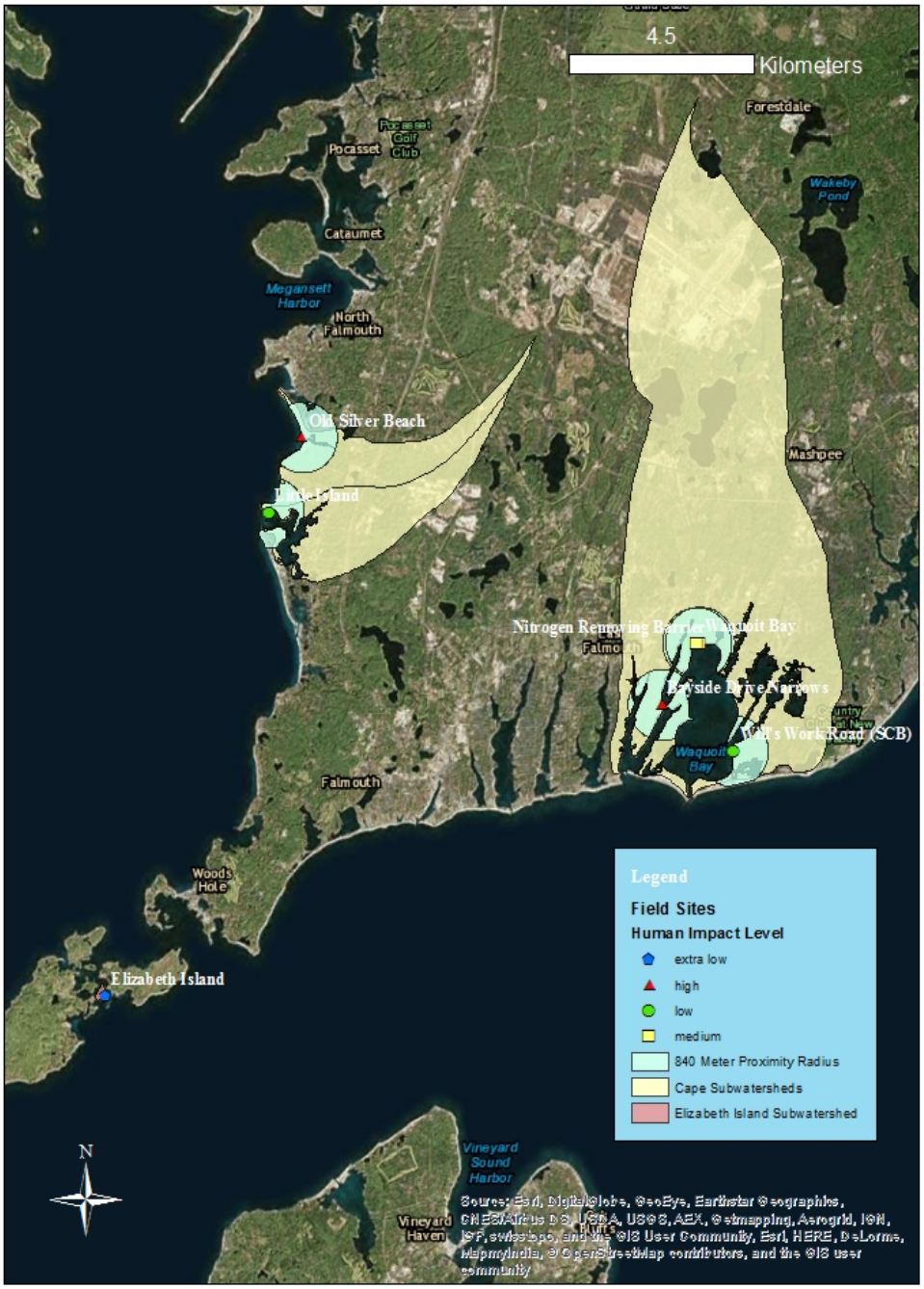


Figure 1. Map of study sites.
Map includes arcGIS information indicating proximity (840 meters around a field site) and watershed land use for each area. Colors and symbols represent if it was a extra low, low, medium, or high human impact level.

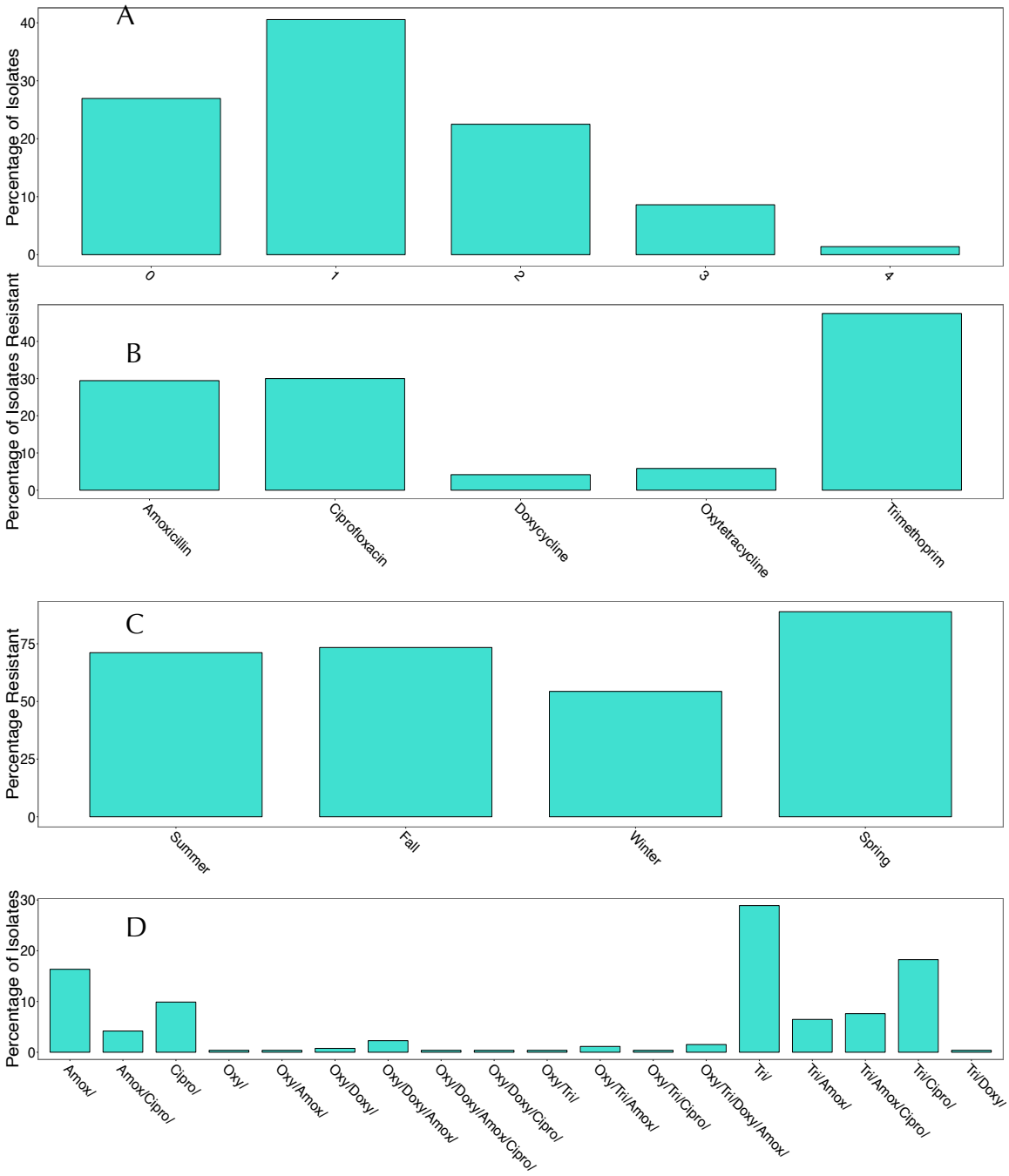


Figure 2. Resistance summary.
A) percentage of isolates resistant to 0,1, or more antibiotics. B) percentage of isolates resistant to each of the five tested antibiotics. Amoxicillin refers to the amoxicillin/clavulanic acid mixture. C) percentage of isolates resistant to at least one antibiotic during each calendar season. D) percentage of resistant data illustrating prevalence of resistance categorization.

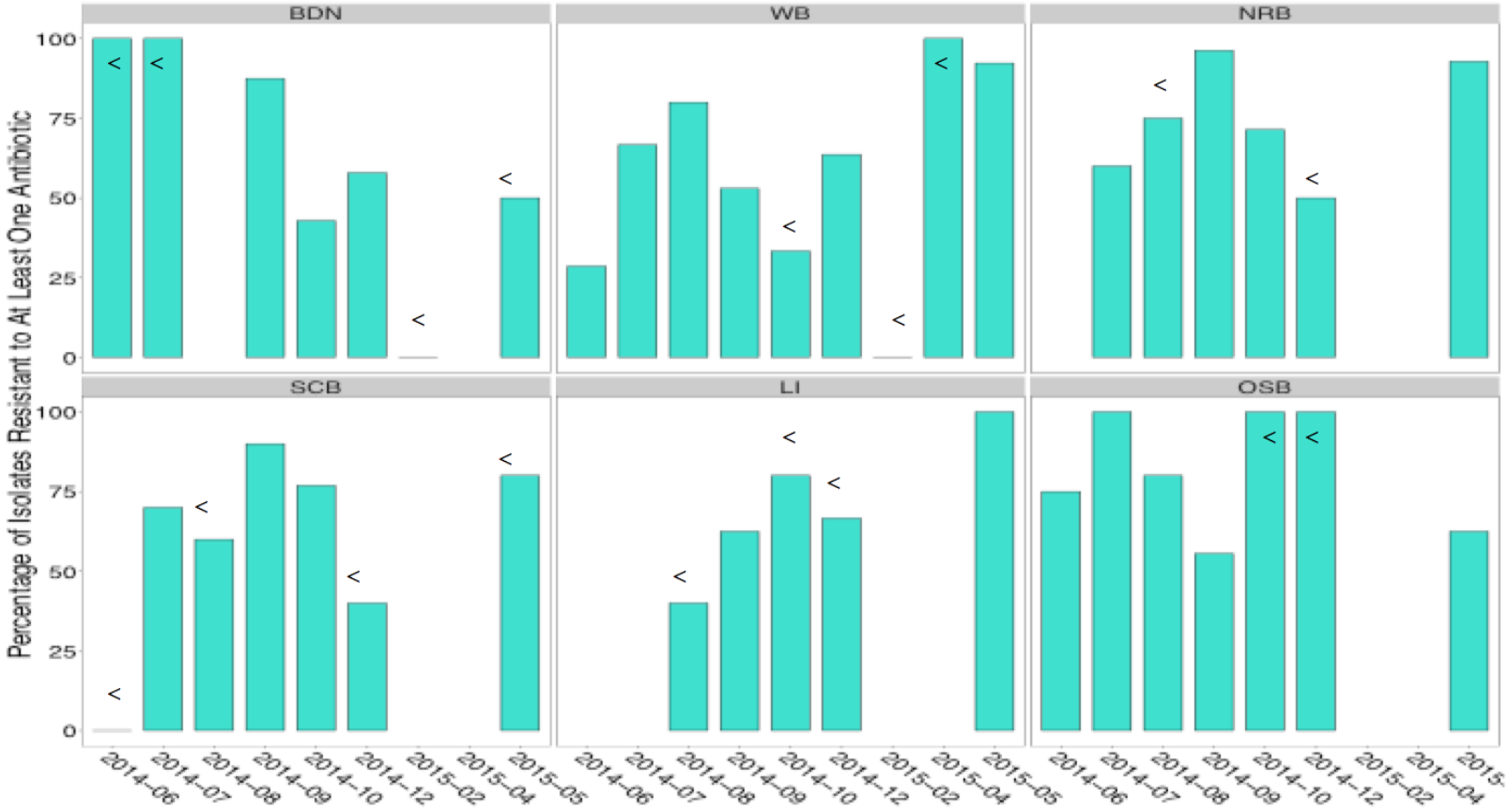


Figure 3. The percentage of isolates resistant to at least 1 antibiotic (y-axis) over time (x-axis).
Each graph indicates a different site. If all isolates from a sampling time point were sensitive to all antibiotics, a black dash is shown. An empty point indicates no isolates from that sampling time were recovered for testing. The less than symbol (<) indicates samples with 5 or fewer isolates.

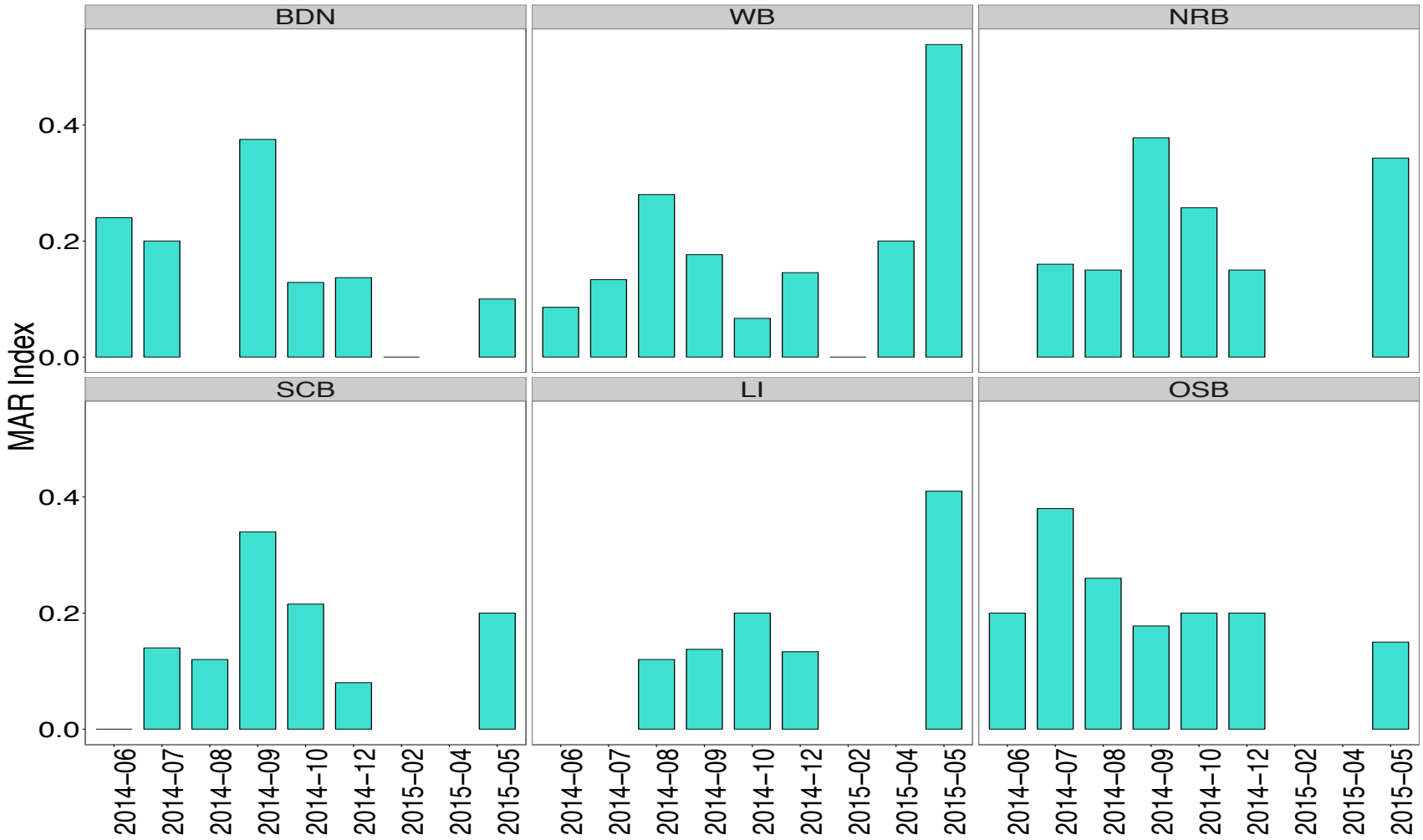


Figure 4. The MAR index (y-axis) value over time (x-axis, also color) by sample location.
If all isolates from a sampling time point were sensitive to all antibiotics, a black dash is shown. An empty point indicates no samples from that sampling time were recovered for testing.

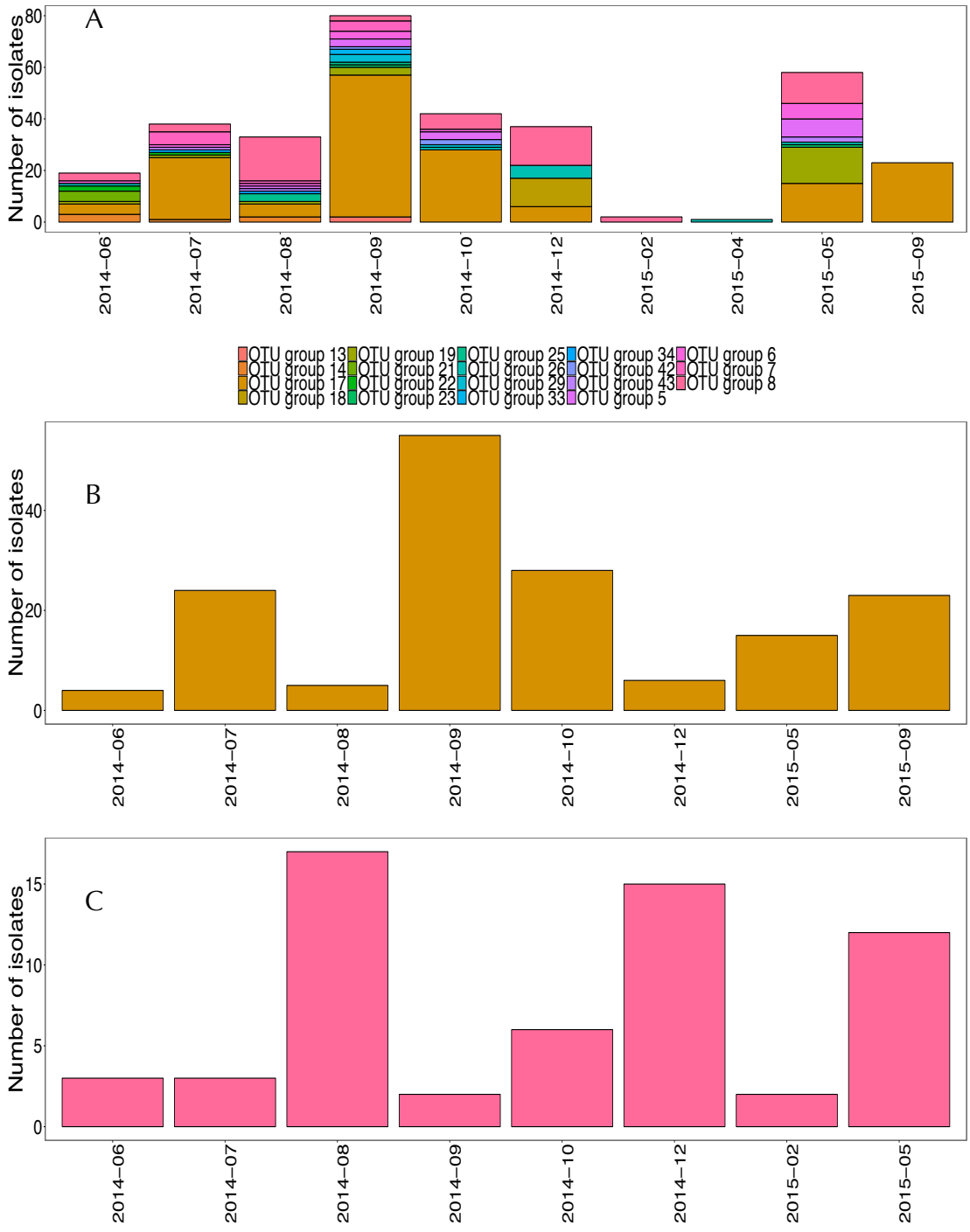


Figure 5. OTU community comparison A) *Vibrio* spp. OTUs over time. The OTU groupings are at 99% identity. OTUs with only one occurrence (singletons) have been excluded. B) OTU Group 17 over time. C) OTU Group 8 over time.

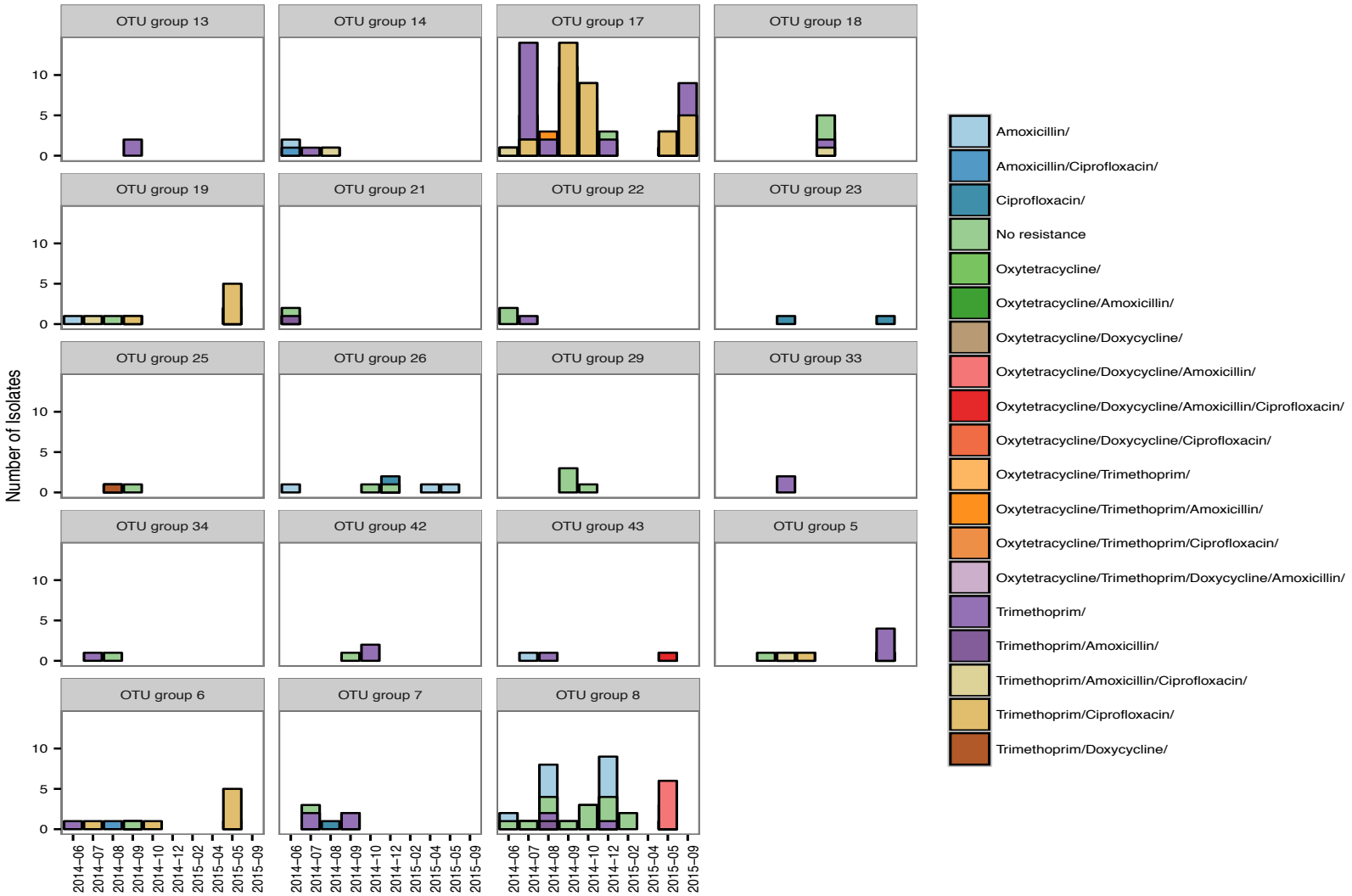


Figure 6. Prevalence of antibiotic resistance types (color) over time (x-axis) by each OTU group (individual graph). The OTU groups indicated here had 2 or more occurrences.

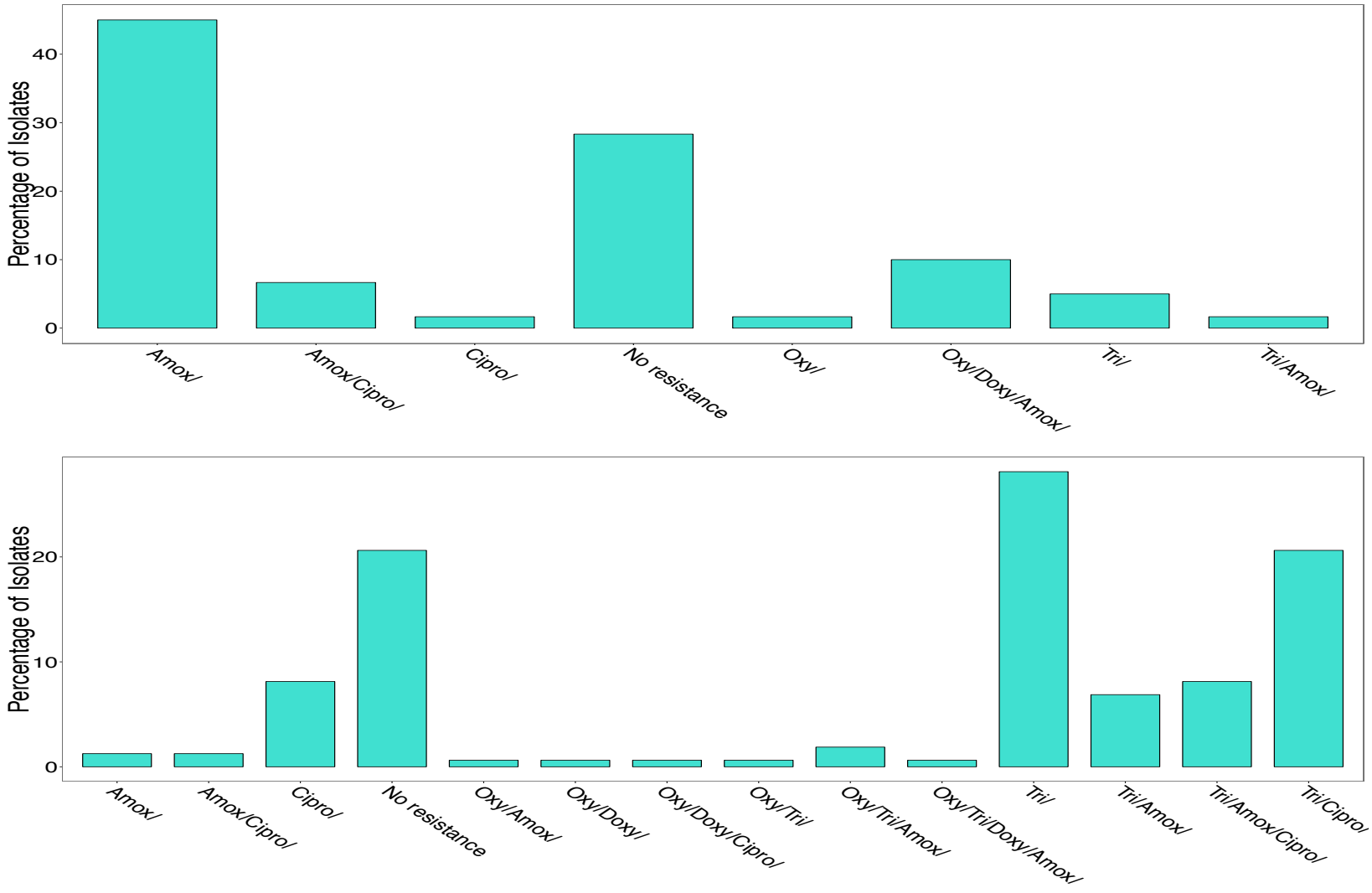


Figure 7. Percentage of resistant isolates illustrating patterns of resistance for A.) OTU Group 8 and B.) OTU Group 17.

Table 1. Description of study sites.
Table indicates the sites, abbreviation, dates, and description of the studied sites.

Site Name	Code	Dates Sampled Day-Month-Year	Human Impact Level	Description	Body of Water	Watershed/ Embayment	Other notes about site
Little Island	LI	6/9/14, 7/29/14, 8/12/14, 9/18/14, 10/27/14, 12/2/14,2/10/15,4/13/15, 5/18/15	Low	Isolated beach in a private neighborhood	Buzzards Bay	West Falmouth Harbor	At times during our study period, the area was considered contaminated as a shellfish ban was in place.
Old Silver Beach	OSB	6/9/14, 7/29/14, 8/12/14, 9/18/14, 10/27/14, 12/2/14,2/10/15,4/13/15, 5/18/15	High	Large and extremely popular beach that has many visitors recreating in and near the water during the summer	Buzzards Bay	Falmouth Old Silver Beach	
Will's Work Road Beach	SCB	6/21/14, 7/31/14, 8/14/14, 9/11/14, 10/30/14, 12/04/14, 02/25/15, 4/11/15, 5/22/15	Low	Small, isolated, hard to access beach near South Cape Beach on Waquoit Bay	Waquoit Bay, Nantucket Sound	Waquoit Bay	Closer to outlet of Bay
Waquoit Bay National Estuarine Research Reserve (WBNERR)	WB	6/21/14, 7/31/14, 8/14/14, 9/11/14, 10/30/14, 12/04/14, 02/11/15, 4/11/15, 5/22/15	Medium	Research beach that is at the top of the Bay, isolated due to being inside WBNERR, the area around WBNERR is mostly residential	Waquoit Bay, Nantucket Sound	Waquoit Bay	At top of Bay; previously studied beach(75–79)

Waquoit Bay National Estuarine Research Reserve Beach Nitrogen Removing Barrier	NRB	6/21/14, 7/31/14, 8/14/14, 9/11/14, 10/30/14, 12/04/14, 02/11/15, 4/11/15, 5/22/15	Medium	Same beach as WB, but located about 150 meters down the beach and is over a nitrogen removing barrier that is placed below the sand	Waquoit Bay, Nantucket Sound	Waquoit Bay	Studied to see if there were differences within the bacterial community within the nitrogen removing barrier
Bayside Drive Narrows Beach	BDN	6/21/14, 7/31/14, 8/14/14, 9/11/14, 10/30/14, 12/04/14, 02/11/15, 4/11/15, 5/22/15	High	High density residential beach in Seacoast Shores neighborhood, main beach for the Seacoast Shores Association	Waquoit Bay, Nantucket Sound	Waquoit Bay	Beach nourished in April 2014
Elizabeth Island SE Gutter, 7 people beach	EI	9/2/15	Very low	Isolated beach on an sporadically inhabited island on a small chain of islands off of Cape Cod and only accessible by boat	Vineyard Sound	Own watershed	Beach sampled on only one occasion

Table 2. Antibiotics and resistance/sensitivity breakpoints used for *Vibrio*-like isolates in this study.

Antibiotic	Abbreviation	Antibiotic Amount (μg)	Antibiotic Class (80)	Mechanism (81, 82)	Sensitive (mm)	Intermediate (mm)	Resistant (mm)	Organism From Source
Amoxicillin/ Clavulanic acid	AMC30	20/10	Penicillins (Beta lactams)	Cell wall synthesis	≥18	14-17	≤13	*Vibrio* spp.(49)
Ciprofloxacin	CIP5	5	Quinolones	DNA gyrase	≥21,	16-20,	≤15	*Vibrio* spp. (49)
Doxycycline	D30	30	Tetracycline	30S ribosomal subunit	≥14	11-13	≤10	*Enterobacteriaceae* (47)
Oxytetracycline	T30	30	Tetracycline	30S ribosomal subunit	≥19	15-18	≤14	*Vibrio* spp. for tetracycline (49)
Trimethoprim	TMP5	5	Trimethoprim	Folic acid metabolism	≥16	11-15	≤10	*Enterobacteriaceae* (47)

Table 3. Recovery information (total isolates) for samples (by location, month, and sample type).

MAR Index included as indicated by Krumperman 1983, calculated by amount of resistance/ (5, which is the number of antibiotics tested, X the number of total isolates tested). Amount of resistance is the total number of antibiotics all isolates were resistant to.

Sample	Sample Location	Month	Sample Type	Amount of Resistance	Total Isolates	MAR Index
BDN2014-06wet	BDN	2014-06	wet	6	5	0.24
BDN2014-07wet	BDN	2014-07	wet	3	3	0.2
BDN2014-09dry	BDN	2014-09	dry	3	2	0.3
BDN2014-09water	BDN	2014-09	water	8	4	0.4
BDN2014-09wet	BDN	2014-09	wet	4	2	0.4
BDN2014-10dry	BDN	2014-10	dry	4	6	0.133333333
BDN2014-10water	BDN	2014-10	water	2	5	0.08
BDN2014-10wet	BDN	2014-10	wet	3	3	0.2
BDN2014-12dry	BDN	2014-12	dry	9	13	0.138461538
BDN2014-12wet	BDN	2014-12	wet	4	6	0.133333333
BDN2015-02wet	BDN	2015-02	wet	0	1	0
BDN2015-05water	BDN	2015-05	water	1	2	0.1
EI2015-09dry	EI	2015-09	dry	14	8	0.35
EI2015-09water	EI	2015-09	water	1	3	0.066666667
EI2015-09wet	EI	2015-09	wet	12	13	0.184615385
LI2014-08dry	LI	2014-08	dry	1	1	0.2
LI2014-08wet	LI	2014-08	wet	2	4	0.1
LI2014-09dry	LI	2014-09	dry	1	4	0.05
LI2014-09water	LI	2014-09	water	9	8	0.225
LI2014-09wet	LI	2014-09	wet	1	4	0.05
LI2014-10dry	LI	2014-10	dry	4	4	0.2
LI2014-10wet	LI	2014-10	wet	1	1	0.2
LI2014-12dry	LI	2014-12	dry	2	3	0.133333333
LI2015-05dry	LI	2015-05	dry	14	6	0.466666667
LI2015-05water	LI	2015-05	water	14	8	0.35
LI2015-05wet	LI	2015-05	wet	13	6	0.433333333
NRB2014-07dry	NRB	2014-07	dry	6	7	0.171428571
NRB2014-07water	NRB	2014-07	water	0	1	0
NRB2014-07wet	NRB	2014-07	wet	2	2	0.2
NRB2014-08wet	NRB	2014-08	wet	3	4	0.15
NRB2014-09dry	NRB	2014-09	dry	9	6	0.3
NRB2014-09water	NRB	2014-09	water	12	6	0.4
NRB2014-09wet	NRB	2014-09	wet	30	15	0.4
NRB2014-10dry	NRB	2014-10	dry	8	5	0.32
NRB2014-10wet	NRB	2014-10	wet	1	2	0.1
NRB2014-12dry	NRB	2014-12	dry	2	2	0.2
NRB2014-12water	NRB	2014-12	water	1	2	0.1
NRB2015-05dry	NRB	2015-05	dry	18	9	0.4

NRB2015-05water	NRB	2015-05	water	2	1	0.4
NRB2015-05wet	NRB	2015-05	wet	4	4	0.2
OSB2014-06water	OSB	2014-06	water	1	3	0.066666667
OSB2014-06wet	OSB	2014-06	wet	7	5	0.28
OSB2014-07dry	OSB	2014-07	dry	7	3	0.466666667
OSB2014-07wet	OSB	2014-07	wet	12	7	0.342857143
OSB2014-08water	OSB	2014-08	water	7	4	0.35
OSB2014-08wet	OSB	2014-08	wet	6	6	0.2
OSB2014-09dry	OSB	2014-09	dry	1	2	0.1
OSB2014-09water	OSB	2014-09	water	6	3	0.4
OSB2014-09wet	OSB	2014-09	wet	1	4	0.05
OSB2014-10wet	OSB	2014-10	wet	1	1	0.2
OSB2014-12wet	OSB	2014-12	wet	1	1	0.2
OSB2015-05water	OSB	2015-05	water	1	1	0.2
OSB2015-05wet	OSB	2015-05	wet	5	7	0.142857143
SCB2014-06water	SCB	2014-06	water	0	1	0
SCB2014-07dry	SCB	2014-07	dry	3	4	0.15
SCB2014-07wet	SCB	2014-07	wet	4	6	0.133333333
SCB2014-08dry	SCB	2014-08	dry	0	1	0
SCB2014-08wet	SCB	2014-08	wet	3	4	0.15
SCB2014-09water	SCB	2014-09	water	7	3	0.466666667
SCB2014-09wet	SCB	2014-09	wet	10	7	0.285714286
SCB2014-10dry	SCB	2014-10	dry	13	11	0.236363636
SCB2014-10wet	SCB	2014-10	wet	1	2	0.1
SCB2014-12dry	SCB	2014-12	dry	1	3	0.066666667
SCB2014-12water	SCB	2014-12	water	1	2	0.1
SCB2015-05water	SCB	2015-05	water	4	4	0.2
SCB2015-05wet	SCB	2015-05	wet	1	1	0.2
WB2014-06water	WB	2014-06	water	3	7	0.085714286
WB2014-07dry	WB	2014-07	dry	4	6	0.133333333
WB2014-07wet	WB	2014-07	wet	2	3	0.133333333
WB2014-08dry	WB	2014-08	dry	11	7	0.314285714
WB2014-08wet	WB	2014-08	wet	3	3	0.2
WB2014-09dry	WB	2014-09	dry	4	6	0.133333333
WB2014-09water	WB	2014-09	water	5	6	0.166666667
WB2014-09wet	WB	2014-09	wet	6	5	0.24
WB2014-10wet	WB	2014-10	wet	1	3	0.066666667
WB2014-12dry	WB	2014-12	dry	2	5	0.08
WB2014-12water	WB	2014-12	water	5	4	0.25
WB2014-12wet	WB	2014-12	wet	1	2	0.1
WB2015-02dry	WB	2015-02	dry	0	2	0
WB2015-04wet	WB	2015-04	wet	1	1	0.2
WB2015-05dry	WB	2015-05	dry	34	11	0.618181818
WB2015-05water	WB	2015-05	water	1	2	0.1

Table 4. Results from PERMANOVA.
Table indicates permutational multivariate analysis of variance (PERMANOVA (63)). These data do not include the Elizabeth Island data because this site was only sampled on one occasion. Bolded and italicized factors indicate significant results. Italics only indicate factors that were pooled.

Dataset	Question	Factors tested (factor type)	Results (p value, variance percentage)
Isolate Matrix	Does human impact affect resistance patterns?	Human Impact (fixed) Month (fixed) Sample Location (random) (nested in Human Impact)	Impact (0.062, 4.9144) Month (0.158, 5.5496) Location (0.414, 1.1559) Impact x. Month (0.295, 5.5958) ***Location x Month (0.001,12.166)*** Residual variance: 23.35%
	Does watershed affect resistance patterns?	Watershed (fixed) Month (fixed) Sample Location (random) (nested in Watershed)	Watershed (0.132, 6.6189) Month (0.232, 5.1202) Location (0.101, 3.5609) Watershed x. Month (0.12, 9.3582) ***Location x. Month (0.001, 10.975)*** Residual variance: 23.25%
RI Count Matrix	Does human impact affect resistance prevalence?	Human Impact (fixed) Month (fixed) Sample Location (random) (nested in Human Impact)	Impact (0.136, 4.3156) ***Month (0.039, 8.6699)*** *Location (0.732, -4.2808)* Impact x. Month (0.615, -3.7195) Residual variance: 17.064% **Pooled Sample Location** Impact (0.272, 3.0356) ***Month (0.009, 9.7047)*** Impact x. Month (0.518, -1.1029) Residual variance: 16.518%
	Does watershed affect resistance prevalence?	Watershed (fixed) Month (fixed)	*Watershed (0.625, -1.2047)* ***Month (0.018, 8.4625)***

	Sample Location (random) (nested in Watershed)	Location (0.322, 2.4734) ***Watershed x. Month (0.027, 10.557)*** Residual variance: 14.973% Pooled watershed ***Month (0.017, 8.4625)*** ***Watershed x. Month (0.026, 10.556)*** Residual variance: 14.973%

Table 5. Results from PERMANOVA for OTU composition data.
This table shows permutational multivariate analysis of variance (PERMANOVA (63)) for the OTU composition data. These data do not include the Elizabeth Island data because this site was only sampled on one occasion. Bolded and italicized factors indicate significant results. Italics only indicate factors that were pooled.

Dataset	Question	Factors tested (factor type)	Results (p value, variance percentage)
OTU Location Month Matrix	Does human impact affect OTU composition?	Human Impact (fixed) Month (fixed)Sample Location (random) (nested in Impact)	Impact (0.5, 3.4295) ***Month (0.002, 24.575)*** *Location (0.638, -8.3803)* Impact x. Month (0.399 ,9.2368) Residual variance: 51.666% **Pooled Location** *Impact (0.587, -5.0338)* ***Month (0.003, 24.118)*** Impact x. Month (0.327, 10.729) Residual variance: 50.982% **Pooled Impact** ***Month (0.001, 24.575)*** Impact x. Month (0.372, 9.2368) Residual variance: 51.666%
	Does watershed affect OTU composition?	Watersheds (fixed) Month (fixed) Location (random) (nested in Watershed)	Watersheds (0.324, 7.3814) ***Month (0.003, 25.166)*** *Location (0.678, -7.9841)* Watershed x. Month (0.125, 20.346) Residual variance: 50.101% **Pooled Location** *Watershed (0.345, 4.7871)* ***Month (0.001, 24.525)*** Watershed x. Month (0.074, 11.994) Residual variance: 49.46%

Table 6. Estimated amount of resistant bacteria ingested.

Resistant bacteria ingested by individuals participating in a given recreational activity by site for water samples collected in September 2014 or September 2015 (for the Elizabeth Island site). The amount of water ingested in each of these activities comes from Leonard et al. 2015. Amount of resistant bacteria includes the estimates for bacteria resistant to 1,2, or 3 antibiotics. Water samples had no isolates that were resistant to four antibiotics.

Recreational activity	Amount of water (ml)	BDN 2014-09	EI 2015-09	LI 2014-09	NRB 2014-09	OSB 2014-09	SCB 2014-09	WB 2014-09
Swimming-children	37	2.1×10^3	1.1×10^4	4.3×10^3	3.6×10^4	1.1×10^4	1.3×10^4	1.3×10^4
Swimming-adults	16	9.1×10^2	4.6×10^3	1.9×10^3	1.6×10^4	4.9×10^3	5.6×10^3	5.7×10^3
Boating	3.7	2.1×10^6	1.1×10^3	4.3×10^2	3.6×10^3	1.1×10^3	1.3×10^3	1.3×10^3
Rowing	3.5	2.0×10^2	1.0×10^3	4.1×10^2	3.4×10^3	1.1×10^3	1.2×10^3	1.2×10^3
Canoeing	3.9	2.2×10^2	1.1×10^3	4.5×10^2	3.8×10^3	1.2×10^3	1.4×10^3	1.4×10^3
Kayaking	3.8	2.2×10^2	1.1×10^3	4.4×10^2	3.7×10^3	1.2×10^3	1.3×10^3	13×10^3
Fishing	3.6	2.0×10^2	1.0×10^3	4.2×10^2	3.5×10^3	1.1×10^3	1.3×10^3	1.3×10^3
Wading/splashing	3.7	2.1×10^2	1.1×10^3	4.3×10^2	3.6×10^3	1.1×10^3	1.3×10^3	1.3×10^3
Diving	9.9	5.6×10^2	2.9×10^3	1.1×10^3	9.7×10^3	3.0×10^3	3.4×10^3	3.5×10^3

Table 7. Estimated amount of resistant bacteria filtered per hour by a variety of shellfish.
The low and high estimates are based on a range of water filtration capabilities for the organisms. Amount of resistant bacteria includes the estimates for bacteria resistant to 1,2, or 3 antibiotics.

Shellfish		Filtration rate		BDN 2014-09	EI 2015-09	LI 2014-09	NRB 2014-09	OSB 2014-09	SCB 2014-09	WB 2014-09
		L/h	ml/h							
Crassostrea virginica	Low filtration	2.04	2040	1.2×10^5	5.9×10^5	2.4×10^5	2.0×10^6	6.3×10^5	7.1×10^5	7.2×10^5
	High filtration	27	27000	1.5×10^6	7.8×10^6	3.1×10^6	2.6×10^7	8.3×10^6	9.4×10^6	9.5×10^6
Mercenaria mercenaria	Low filtration	0.3	300	1.7×10^4	8.7×10^4	3.5×10^4	2.9×10^5	9.2×10^4	1.0×10^5	1.1×10^5
	High filtration	3.6	3600	2.1×10^5	1.0×10^6	4.2×10^5	3.5×10^6	1.1×10^6	1.3×10^6	1.3×10^6
Mya arenaria	Low filtration	1.7	1700	9.7×10^4	4.9×10^5	2.0×10^5	1.7×10^6	5.2×10^5	5.9×10^5	6.0×10^5
	High filtration	7.4	7400	4.2×10^5	2.1×10^6	8.6×10^5	7.3×10^6	2.3×10^6	2.6×10^6	2.6×10^6

CHAPTER 4 Heterotrophic marine bacteria from Cape Cod beaches harbor prevalent amounts of antibiotic resistance

INTRODUCTION

Antibiotic resistance is a growing problem with a projected ten million deaths in 2050 caused by antibiotic resistance (1). The global, cumulative economic cost of antibiotic resistance from 2016 to 2050 would be 100 trillion dollars (1). Although antibiotic resistance in humans is primarily considered to arise from use in clinics and hospitals, recently attention has shifted to the interplay of humans, animals, and the environment, often called the "One Health Concept", suggesting that understanding the contribution of these other avenues is critical to quantifying and elucidating the problem and then employing relevant solutions. To date, a number of studies have quantified antibiotic resistant bacteria in humans and in animals, but the role of the environment lags behind in research, especially the marine environment. The ocean is a vast space and the majority of the human population—4 billion people (66%) in 1998—live within 400 km of a coast (2). Humans routinely interact with the ocean through food consumption and recreation, and the beach is one venue where these activities occur, illustrating a potential transfer area.

Heterotrophic bacteria—bacteria that fulfill their carbon needs by ingesting organic carbon—are prevalent in the ocean (3, 4). These native bacteria may serve as a reservoir of antibiotic resistance (AR) genes in the marine environment, either as a natural condition or as the result of acquiring them from bacteria that have been introduced to the marine environment through anthropogenic activities. Natural environments are the origin of most antibiotics used in the clinical environment, and it is therefore unsurprising to also find resistance in these regions. Together, this makes the ocean and beaches a potential venue

for transfer of resistance between anthropogenic and natural bacteria, and into humans through recreation or food consumption.

Previous research has found resistance in heterotrophic bacteria in the ocean. One of the first studies illustrated that resistant bacteria could be found in seawater from coastal and offshore samples and from surface to deep water samples (8200 m) (5). A study examining the estuarine Lake Gardno in Poland showed that resistance was present and prevalence varied largely based on antibiotic (6). A study in Algiers, Algeria illustrated that beaches with anthropogenic inputs had higher levels of resistance compared to less impacted sites (7). A study in São Vicente, Brazil showed a similar trend between beaches of high, medium, and low pollution (8). Mudryk's studies have also illustrated a similar trend within sand on beaches, showing higher resistance on a beach with high human activity in Sopot, Poland compared to an isolated beach in a national park near Czolpino, Poland (9, 10). All of these prior studies have firmly established that antibiotic resistant bacteria are present at marine beaches, particularly where anthropogenic activity is high; but research regarding the diversity and persistence of AR over temporal and seasonal scales is still lacking.

This study seeks to examine the diversity and persistence of antibiotic resistance in marine heterotrophs from sand and water over temporal, spatial, and human impact scales at the beach. The hypotheses are that beaches with greater human activity have higher occurrences of resistance, and sand samples have higher levels of resistance. Terrestrial sediments are known to have a greater bacterial density (11), which can promote gene transfer and has often been a source of new antibiotic discoveries (12). Winter is expected

to have less resistance overall than spring, summer, or fall because of reduced temperatures, bacterial numbers and human activity. Estimates of the number of antibiotic resistant bacteria filtered by shellfish or ingested by humans through recreation in water were calculated to better understand the potential for human exposure to these organisms in the marine environment. The results are discussed with respect to the *Vibrio* study reported on in Chapter 3, to synthesize what has been learned from both efforts.

METHODS

Site description (Chapter 3,Table 1) and map (Chapter 3, Figure 1), field sampling, and sample processing followed the same methods found in Chapter 3.

Cultivation of heterotrophic bacteria

Heterotrophic bacteria were cultured by direct plating of water and/or sediment-PBS mixture onto marine agar 2216 media (Difco). Serial dilutions were completed and a variety of samples were plated from the original sample to 10^{-4}. 100 µl of sample were plated. One plate was chosen for each sample, based ideally on a plate with isolated, easily countable colonies. For each plate, the total colony number was counted, and up to three colonies of each morphology type on a given plate were picked. Picked colonies were grown in seawater broth and 800 µl of the culture was added to 200 µl of sterile 80% glycerol and stored at -80°C as sample stocks.

Antibiotic resistance testing through minimum inhibitory concentration testing

Antibiotic resistance testing was completed using the minimum inhibitory concentration (MIC) method (13). This method allows for testing of a wide variety of concentrations to obtain a more detailed understanding of MIC. This method is especially

convenient for bacteria that are not well-studied in the clinical environment and do not have established clinical procedures, such as marine bacteria (14).

For this method, bacteria were tested for sensitivity to four antibiotics (amoxicillin, ciprofloxacin, doxycycline, erythromycin) at nine different concentrations (0.1, 1, 2, 4, 8, 16, 32, 64, 100 µg/ml). Antibiotics were allowed to equilibrate to room temperature for two hours to ensure more accurate weighting. Amoxicillin (Research Products International) was dissolved in DMSO (Fischer) whereas ciprofloxacin (Alfa Aesar), doxycycline (Alfa Aesar, Research Products International), and erythromycin (MP Biomedicals) were dissolved in sterile distilled water. Erythromycin at high concentrations often needs ethanol as a solvent, but for the concentrations used here, water worked well (Table 1). All subsequent dilutions were accomplished with seawater media and were prepared at double the desired final concentration for dispensing into the culture plates, since they would be diluted by the addition of the bacteria cultures. 50 µl of each concentration was placed into a well of a sterile, non-tissue culture 96 well plate (USA Scientific). Amoxicillin, ciprofloxacin, and doxycycline plates were placed in a freezer for use within two weeks. Erythromycin plates were made fresh each time and stored in foil until use because freezing seemed to reduce the effectiveness of the antibiotic (personal observation).

Established bacterial controls for assessing plate to plate variability did not exist due to the type of bacteria being tested (marine heterotrophs), lack of species identification, and the use of seawater broth for growth. Therefore, controls for the testing process were created using selected marine heterotroph cultures. Cultures were chosen

randomly and tested multiple times to ensure reproducibility of MIC values. The final chosen controls had reproducible MIC values and grew well. A bacterial control was included on every plate; and if it did not perform within the standard results, the plate was considered a failure. More information on the isolates used for controls is available in Table 2.

For testing, bacterial isolates were grown from glycerol stocks in seawater broth overnight, shaking at 25 °C. Plate setup can be seen in Figure 1, where each row represents one tested bacterial isolate. 50 µl of seawater media was added to the plates in Column 10 to serve as a positive growth control well. 100 µl of seawater media was added to the plates on Column 12 as a sterile (negative) control. Column 11 remained empty. Each bacterial isolate was adjusted to the MacFarland turbidity standard of 0.5 (Remel); and 50 µl was added to wells 1-10. Once all plates were innoculated, they were placed in an incubator at 25 °C without shaking for 20 hours with a damp paper towel to help maintain moisture levels. A successful test required the plate bacterial MIC control to be within limit, the growth control to have growth, and the sterile control to not have growth. If these conditions were met, the MIC was measured using the definition from Wiegland as "MIC is the lowest concentration of the antimicrobial agent that inhibits visible growth of the tested isolate as observed with the unaided eye" (13).

Determination of resistance

Once the MIC values were recovered, a determination of resistance breakpoints for marine heterotrophs was necessary. To do this, resistance was defined by examining the CLSI method to determine all possible definitions of resistance and sensitivity for each

antibiotic. Then, the most stringent option (highest minimum inhibitory concentration) was chosen to define resistance for each antibiotic. Therefore, these values should be a fairly conservative estimation of resistance**.** Breakpoints are given in Table 1 and are represented in Figure 2.

Other methods

The arcGIS protocol followed the same methods explained in Chapter 3. The estimations for shellfish filtration and human ingestion also followed the same methods as explained in Chapter 3, with the exception that October 2014 samples were used for the estimates. Statistical analysis was the same as conducted in Chapter 3.

RESULTS

1837 bacterial isolates were collected over the nine sampling occassions at the six sites. In order to streamline the testing, one month was chosen to represent each season - August, October, February, and May. This resulted in 952 bacterial isolates, of which 877 were successfully tested for resistance (Table 3). The remainder (75) were not successful due to inability to regrow after time in freezer, measurements for fewer than four antibiotics, or growth and/or contamination problems.

The four antibiotics tested were chosen to represent a variety of antibiotic groups and structural targets. MIC values varied by antibiotic and are shown in Figure 3, along with resistance breakpoints. Isolate amoxicillin MIC values occurred over a wide range between 1-8 µg/ml with a large peak at >100 µg/ml. Ciprofloxacin MICs tended to be below 8 µg/ml, but the highest number of isolates had MIC values of 1 µg/ml. Doxycyline

isolate MIC values peak at 64 µg/ml with another large peak at >100 ug/ml. Erythromycin isolate MICs exhibit a bell-shaped distribution with a peak at 16 ug/ml.

Antibiotic resistance of heterotrophic marine bacteria

Of the 877 isolates tested, 95% (837 isolates) were resistant to at least one antibiotic and 62% (544 isolates) were resistant to more than 1 antibiotic (Figure 3A). 11% (104 isolates) were resistant to all four antibiotics. The amount of resistance varied by antibiotic, with doxycycline having the largest number of resistant isolates (90.7%, 796 isolates; Figure 3B). The other three antibiotics had lower and more similar levels of observed resistance (Figure 3B): ciprofloxacin 42.0% (368 isolates), amoxicillin 36.0% (316 isolates) and erythromycin 29.6% (260 isolates). Resistance to at least one antibiotic by month showed August with 92.3% (120 resistant isolates/ 130 total), October 93.9% (232 resistant isolates/ 247 total), February 93.7% (150 resistant isolates/160 total), May 98.2% (287 resistant isolates / 292 total), and September 100% (48 resistant isolates out of 48 total, only Elizabeth Island was sampled) (Figure 3C).

The categorization of resistance (Figure 4) illustrates the types of resistance seen in the 877 samples. Doxycycline resistance was most prevalent (29.5% of the samples), followed by ciprofloxacin/doxycycline in 13.6% of the samples, resistance to all antibiotics in 11.9%, and amoxicillin/doxycycline in 10.7%. Resistances that were infrequently seen (<1%) were erythromycin, ciprofloxacin/erythromycin, amoxicillin, amoxicillin/erythromycin, amoxicillin/ciprofloxacin, and amoxicillin/ciprofloxacin/erythromycin.

Antibiotic resistance is illustrated by site, season, and sample type in Figure 5. Resistance of isolates to at least one antibiotic was prevalent for each particular site/season/type categorization (Figure 5). The lowest value observed was no resistance to at least one antibiotic for two groups- OSB 2014-08 wet and SCB 2014-08 dry. However, these sites are based on an isolate number of five or less (Figure 5). One sample (OSB 2015-02 wet) did not have any recovered isolates.

Statistical analysis

Permutational multivariate analysis of variance (PERMANOVA) was used to assess statistical significance of two main questions- 1) Does human impact affect resistance patterns/prevalence? and 2) Does watershed affect resistance patterns/prevalence? The isolate matrix was used examine resistance patterns with respect to human impact and showed that month (accounting for 6.9% of the variance), sample type (4.6 % of the variance), and the interaction of month and type (8.7% of the variance) were significant in affecting resistance prevalence (Table 4). Impact and location were not significant (Table 3). In the second test to determine resistance patterns by watershed, watershed was not significant (Table 4). Month (7.1% of the variance) and the interaction of month and type (8.7% of the variance) were significant, even when location was pooled as a factor.

To examine prevalence, the resistant isolate (RI) count matrix was used. Here, this showed month as the only significant factor (7.1% of the variance) in answering the question of human impact, even when other factors were pooled (Table 4). To examine resistance prevalence by watershed, initially month showed significance, but dropped out when factors were pooled, leaving no factors with significance.

Distance-based linear models (DISTLM) were used to compare resistance data with environmental data of three types: 1) land use in an 840 meter radius around the sites (called proximity land use), 2) land use in the watershed, and 3) environmental variables collected at the time of sampling. 840 meters was chosen as the radius because it allowed for the largest amount of space without overlap from sites (barring the WB and NRB that are on the same site). For proximity land use, the percentage of the population male was the variable that explained the most variance, although it was a very small amount of the total variability (0.092%). For watershed land use, the best model was a one variable model with percentage of urbanized land use as the explanatory variable with 1.13% of the variability. A close second was the mean median age of the watershed explaining 1.08% of the variability. For the environmental variables, the best model had a single variable of dissolved oxygen with 3.2% of the total variation. Two close models were one with amount of sunlight and dissolved oxygen (4% total variation) and amount of sunlight and temperature (4% total variation).

Ingestion and filtration estimates

Ingestion estimates of resistant bacteria for humans participating in recreational activities were made from the amount of resistant bacteria per milliliter determined from plate counts and previous literature estimates of how much water a person ingests during various activities. SCB was the site with the highest values of resistant cells (2×10^4 resistant bacteria per ml) and LI had the lowest (1.9×10^3 resistant bacteria per ml).

Swimming is the activity that results in ingestion of the most water. Children ingest more than two times the amount of water (37 ml) ingested by adults (16 ml). Rowing has

the lowest amount at 3.5 ml. Overall, estimates of bacteria ingested for each activity had a wide range from $6.6 \times 10^3 - 7.3 \times 10^5$ resistant cells ingested per activity (Table 5).

Filtration estimates were also produced for three common shellfish in the area. Using published values of low and high filtration rates, estimates were made of the amount of resistant bacteria a shellfish would filter in one hour. *Mercenaria mercenaria* has the lowest filtration rate of 300 ml/hour whereas *Crassostrea virginica* has the highest rate at 2700 ml/h. Resistant cells filtered in one hour ranged from 5.6×10^5 to 5.3×10^8 (Table 6).

DISCUSSION

Resistance is widespread in studied heterotrophic marine bacteria.

In this study, results obtained using conservative measures of resistance showed that antibiotic resistance was prevalent in the studied samples, with 95% of isolates resistant to at least one antibiotic and 12% resistant to all four antibiotics. These results are slightly higher, but in general are within the results from previously published literature. At marine beach sites in Brasil, Cardoso de Olivera et al. (8) found resistance to at least one antibiotic in 35.3%, 77.7%, and 80.6% of samples from three different sites. Another study conducted at an isolated marine beach that humans rarely visit found 70% of isolates resistant to at least one antibiotic (10). As a comparison with a putatively "un-impacted" environment, 90% of the bacterial isolates from a study of deep terrestrial subsurfaces were found resistant to at least one antibiotic, despite the sites being 170-259 meters below the surface of the ground (15).

In addition, it seems likely that the high amounts of resistance found in this study may indicate that antibiotic interactions in the coastal ocean are common and that the coastal ocean has reached exposure saturation with antibiotic input from both anthropogenic and natural inputs. The lack of human impact differences could be due to this saturation.

Resistance varies by antibiotic.

Since the amount of resistance is dependent on the antibiotics studied and their mechanisms of action (6), this study examined four antibiotics with different mechanisms of action (Table 2). Doxycycline resistance was prevalent with a majority of the isolates resistant to this antibiotic (Figure 4B). Ciprofloxacin and amoxicillin resistance were lower, 41.9% and 36.0% respectively. Erythromycin has the lowest value with 29.6%. Previous studies indicate that in Lagos Lagoon, Nigera, resistance was measured as 10-29% for amoxicillin, no resistance for ciprofloxacin, and 0-25% for erythromycin, with fluctuations depending on the site (16). In sand at an isolated marine beach, resistance to amoxicillin was 23%, ciprofloxacin 18%, doxycycline 3%, and erythromycin 18% (10). For Brazilian beaches in São Vicente, resistance prevalence values varied depending on the individual beach and ranged from 0 to 20.8% for amoxicillin, 0 to 25% for ciprofloxacin, and 0 to 33.3% for erythromycin (8). For doxycycline, 20% resistance was found in Gdansk Deep (17) while 7% was found at a recreational marine beach on the Baltic Sea (9) and 71% isolates were resistant to doxycycline in the estuarine Lake Garno (6). For erythromycin, Baya et al. (18) studied sewage effluent, outfall diffusers, and coastal waters, with an average value of 27.5% resistance to erythromycin; but individual sites varied from 0 to

67.8% with the lowest value at the coastal ocean and the highest value in the effluent. In general, the results from the current study fall well within the ranges seen in other marine work, though the value for doxycycline is higher than previous studies. One possible factor that might influence the doxycycline resistance prevalence is that Lyme disease is fairly common on Cape Cod, and New England in general, and doxycycline is the primary antibiotic used to treat the infection.

Mechanism of resistance may be driving high values of resistance for antibiotics.

In this study, resistance as a phenotypic response was measured, but no information about the actual mechanism of resistance was obtained. Understanding the mechanism would identify whether the resistance is gene-based or related to cellular mechanisms that are are not specific for a particular antibiotic (e.g. efflux pumps). All antibiotics used in this study were selected because they should be effective on either Gram positive or Gram negative bacteria. Gram negative bacteria are intrisically resistant to some antibiotics (e.g. vancomycin); and many marine bacteria are Gram negative, such as Pelagibacterales (otherwise known as SAR11 (19)).

Previous research has shown that marine bacteria (the order Vibrionales) readily produce inhibitory molecules and are resistant to them (20). Research by Cordero showed that within the family Vibrionaceae, few bacteria produce antibiotics whereas most are resistant (21). The researchers took this as evidence of collaboration within the bacterial group, where many could benefit from the production of a compound without expending resources. This study adds further evidence for the existence of antibiotic resistance in the ocean, and therefore also suggests antibiotics are likely present. In this light, it seems

reasonable to see antibiotic production and resistance as part of the larger evolutionary interests and benefits to the bacterium.

Bacteria have efflux pumps that pump chemicals out of the cell and can be general or specific in transportation processes. There are currently five main efflux pump families: ATP-binding cassette (ABC), major facilitator superfamily (MFS), resistance/nodulation/cell divison (RND), small multidrug resistance (SMR), and multidrug and toxic compound extrusion (MATE) (22, 23). These efflux pumps allow for movement of antibiotics (and other chemicals) out of the cell, but may also meet a variety of other cellular survival needs including: virulence, pathogencity, quorum sensing, detoxification, and biofilm formation (22, 24). With this in mind, efflux pumps are highly conserved, present in all living things, and may be considered "evolutionarily ancient elements" (22). Efflux pumps are believed to be prevalent in bacterial genomes. One estimate is that efflux pumps represent 10% of all transporters in a cell (22). Transporters are genes that move materials in the cell, of which efflux pumps are one such transporter type. Another estimate is that 5-10% of all genes deal with transport (25).

Multi-drug efflux pumps are problematic in the clinic because they allow for resistance to a variety of antibiotics and increase difficulty in treating resistant infections (26). In the marine environment, treatment for infections is less prevalent than in the clinics. However, a metagenomic study found that a dominant mechanism of resistance in environmental samples, including some from the ocean, was through efflux pumps (27). Further, efflux pumps are effective with Gram negative bacteria (23) and the marine bacterial community is dominant with Gram negative bacteria (19). Therefore, it may be

that the large amounts of resistance seen here in the coastal ocean could be due to prevalence of efflux pumps. This hypothesis would require research into uncovering specific resistance mechanisms in marine bacteria.

Ingestion and filtration estimates show interactions with resistant bacteria are common.

Like in Chapter 3, ingestion and filtration estimates from this study indicate that humans and shellfish readily interact with marine bacteria. Not surprisingly, there are more interactions with resistant heterotrophs per ml of water than resistant *Vibrio*-like bacteria. Compared to the total number of bacteria ingested, these resistant heterotrophic, culturable bacteria represent between 0.02 to 0.2% of the total bacteria present per milliliter of water (assuming 10^7 cells per milliliter). While this is higher than the amount for *Vibrio*-like bacteria, it is still an extremely low percentage. Further research needs to be done to determine if this low comparative abundance reduces potential risk.

In this study, the fact that month and season were significant in structuring heterotrophic antibiotic resistance supports the hypothesis that changes in bacterial community composition may influence resistance patterns. Because heterotrophic bacteria are likely a diverse collection of species, bacterial groups may be differently affected by particular land use or environmental characteristics, which would be masked by examining them as a large, homogenous group. Sequencing to identify the isolates may provide insights to whether there are patterns of resistance related to the types of bacteria present.

In contrast, human impact, land use, and environmental variables accounted for little of the variation observed in antibiotic resistance patterns of heterotrophic marine

bacteria. Other studies have found differences in levels of antibiotic resistance related to human impact in the marine environment. Three studies on beaches in Brazil, in Algeria, and in Poland found greater resistance on beaches with increased human influence (7–10). Miller et al. (28) showed that in Antarctica, there was increased AR in areas near to the Palmer Station, and that multiple resistance was low in pristine environments.

It may be that at the sites chosen for this study, the proposed impacts do not have enough variation to produce a difference in amounts of antibiotic resistance or types of resistance, and that sites more directly impacted by point source pollution would be needed to see a measurable difference. Impact in this area may be so widespread that a sufficiently pristine control (such as Antartica or deep sea marine sediments) is not available within the region.

Alternatively, it may be that resistance itself is more prevalent in the marine environment than previously thought. Other studies in isolated regions have found higher than anticipated amounts of antibiotic resistance. At the Lucky Strike hydrothermal vent field, Farias et al. (29) found that resistance is widespread in the marine environment regions without human impact. In studying Arctic fjords, there was a higher prevalence of resistance than anticipated, and higher values in sediment compared to water (30). In Gdansk Deep in the Baltic Sea, Mudryk et al. (17) found that many bacteria carried multiple antibotic resistances. Finally, Sizemore and Colwell (5) found resistant bacteria at nearshore and deepsea samples, and that coastal areas lended themselves to having more resistant bacteria in terms of samples, but not by percentage.

Antibiotic resistance in Vibrio and heterotrophic bacteria

This study, and the study on *Vibrio* in Chapter 3, evaluated antibiotic resistance at the same sites. These heterotrophic and *Vibrio*-like isolates have been functionally defined by the isolation media used. Marine agar 2216 was used for heterotrophs and thiosulfate citrate bile salts sucrose (TCBS) for *Vibrio*-like bacteria. Disk diffusion was used for the *Vibrio*-like bacteria; the minimum inhibitory method was utilized for the heterotrophs. The heterotrophs represent one large group of bacteria, whereas as the *Vibrio*-like group represents a specific group within the larger framework. While the studies did not use the same methodology to test resistance, general similarities and differences can be drawn from these results.

In terms of resistance, heterotrophs were more resistant on the whole (95% resistant to at least one antibiotic) compared to *Vibrio*-like bacteria (73% resistance to at least one antibiotic). Resistance to all antibiotics tested was more prevalent for heterotrophs, with 11.9% of isolates resistant to all four tested antibiotics, whereas no *Vibrio*-like bacteria were resistant to all five of the tested antibiotics. For both groups, there were small variations by location, but no significance. Temporally resistant *Vibrio*-like bacteria were less likely to be found in the winter, whereas the recovery of heterotrophs stayed fairly constant throughout the year. Finally, there were three antibiotics that were tested for both groups: amoxicillin, ciprofloxacin, and doxycycline. Amoxicillin and ciprofloxacin resistance prevalences were fairly close between groups, while doxycycline resistance was very high in heterotrophs and very low in *Vibrio*-like bacteria. These antibiotics do not have similar chemical structures (31). This illustrates the potential differences in resistances present within different marine bacteria, and more needs to be understood

about mechanisms of action for these resistances to understand what may be driving this difference. One potential option might be that for the heterotrophs, the mechanism that provides resistance to doxycycline may confer a benefit that helps them survive. This may not be the case for the *Vibrio*-like bacteria, and could even exert a cost that reduces their ability to survive.

Environmental variables more readily explained the resistance variations for *Vibrio* (12.93%) then for heterotrophs (3.26%). Since *Vibrio*-like bacteria are a more specific group, it may be easier to observe community structure and its drivers, whereas heterotrophs are a diverse, mixed group, masking potential differences. These differences may be explored further through 16S ribosomal sequencing of the heterotrophs, allowing testing for different genera or species.

CONCLUSION

Taken together, results from this study and others suggest that resistance is prevalent in the coastal marine environment , and that high levels of resistance may be due to resistance serving other critical roles in the survival of marine bacteria. While all of the studies discussed here tested different types of bacteria and different antibiotics, they ultimately paint an overall picture of widespread and abundant antibiotic resistance in environments with and without anthropogenic impact. Furthermore, resistance is prevalent in environmental groups of bacteria. While they do not generate the immediate public health concerns that pathogenic bacteria do, the fact that they grow and disperse means that they can propogate and share resistance with other environmental bacteria as well as pathogenic bacteria. This result suggests a need to examine environmental areas

for emerging resistances that may be transferred to pathogens in order to understand potentially upcoming clinical resistances. From an ecological perspective, it encourages renewed interest in the role that mechanisms of resistance play, why they are so prevalent in the marine enviornment, and what factors may be driving this prevalence.

References

1. **O'Neill J**. 2016. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations.
2. **Hinrichsen D**. 1998. The Coastal Population Explosion. Next 25 Years Glob. Issues 27–29.
3. **Morris RM**, **Rappé MS**, **Connon SA**, **Vergin KL**, **Siebold WA**, **Carlson CA**, **Giovannoni SJ**. 2002. SAR11 clade dominates ocean surface bacterioplankton communities. Nature **420**:806–810.
4. **Duarte CM**, **Regaudie-de-Gioux A**, **Arrieta JM**, **Delgado-Huertas A**, **Agustí S**. 2013. The Oligotrophic Ocean Is Heterotrophic. Ann. Rev. Mar. Sci. **5**:551–569.
5. **Sizemore RK**, **Colwell RR**. 1977. Plasmids carried by antibiotic-resistant marine bacteria. Antimicrob. Agents Chemother. **12**:373–82.
6. **Mudryk ZJ**. 2002. Antibiotic Resistance among Bacteria Inhabiting Surface and Subsurface Water Layers in Estuarine Lake Gardno. Polish J. Environ. Stud. **11**:401–406.
7. **Alouache S**, **Kada M**, **Messai Y**, **Estepa V**, **Torres C**, **Bakour R**. 2012. Antibiotic Resistance and Extended-Spectrum β-Lactamases in Isolated Bacteria from Seawater of Algiers Beaches (Algeria). Microbes Environ. **27**:80–86.
8. **Cardoso de Oliveira AJF**, **Ranzani de França PT**, **Pinto AB**. 2009. Antimicrobial resistance of heterotrophic marine bacteria isolated from seawater and sands of recreational beaches with different organic pollution levels in southeastern Brazil: evidences of resistance dissemination. Environ. Monit. Assess. **169**:375–84.
9. **Mudryk ZJ**. 2005. Occurrence and distribution antibiotic resistance of heterotrophic bacteria isolated from a marine beach. Mar. Pollut. Bull. **50**:80–86.
10. **Mudryk Z**, **Perliński P**, **Skórczewski P**. 2010. Detection of antibiotic resistant bacteria inhabiting the sand of non-recreational marine beach. Mar. Pollut. Bull. **60**:207–14.
11. **Bay C**, **Breuker A**, **Köweker G**, **Blazejak A**, **Schippers A**. 2011. The deep biosphere in terrestrial sediments in the Chesapeake Bay area, Virginia, USA. Front. Microbiol. **2**:1–13.
12. **Lewis K**. 2013. Platforms for antibiotic discovery. Nat. Rev. Drug Discov. **12**:371–87.
13. **Wiegand I**, **Hilpert K**, **Hancock REW**. 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nat. Protoc. **3**:163–175.
14. **Clinical and Laboratory Standards Institute**. 2014. M100-S24: Performance Standards for Antimicrobial Susceptibility Testing, 24th ed. Wayne, PA.
15. **Brown MG**, **Balkwill DL**. 2009. Antibiotic resistance in bacteria isolated from the deep terrestrial subsurface. Microb. Ecol. **57**:484–493.
16. **Agwu O**. 2013. Antibiotic resistance among heterotrophic bacteria in Lagos Lagoon, Nigeria. African J. Aquat. Sci. **38**:331–336.
17. **Mudryk Z**, **Skórczewski P**. 1998. Antibiotic resistance in marine neustonic and planktonic bacteria isolated from the Gdansk Deep. Oceanologia **40**:125–136.

18. **Baya AM**, **Brayton PR**, **Brown VL**, **Grimes DJ**, **Russek-Cohen E**, **Colwell RR**. 1986. Coincident plasmids and antimicrobial resistance in marine bacteria isolated from polluted and unpolluted Atlantic Ocean samples. Appl. Environ. Microbiol. **51**:1285–1292.
19. **Giovannoni SJ**. 2017. SAR11 Bacteria: The Most Abundant Plankton in the Oceans. Ann. Rev. Mar. Sci. **9**:231–255.
20. **Long RA**, **Azam F**. 2001. Antagonistic interactions among marine pelagic bacteria. Appl. Environ. Microbiol. **67**:4975.
21. **Cordero OX**, **Wildschutte H**, **Kirkup B**, **Proehl S**, **Ngo L**, **Hussain F**, **Le Roux F**, **Mincer T**, **Polz MF**. 2012. Ecological Populations of Bacteria Act as Socially Cohesive Units of Antibiotic Production and Resistance. Science (80-.). **337**:1228–1231.
22. **Martinez JL**, **Sánchez MB**, **Martínez-Solano L**, **Hernandez A**, **Garmendia L**, **Fajardo A**, **Alvarez-Ortega C**. 2009. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiol. Rev. **33**:430–449.
23. **Li X-Z**. 2016. Antimicrobial Resistance in Bacteria: An Overview of Mechanisms and Role of Drug Efflux Pumps, p. 131–164. *In* Elkins, X-Z, Elkins, CA, Zgurskaya, HI (eds.), Efflux-Mediated Antimicrobial Resistance in Bacteria. Springer International Publishing, Switzerland.
24. **Sun J**, **Deng Z**, **Yan A**. 2014. Bacterial multidrug efflux pumps : Mechanisms , physiology and pharmacological exploitations. Biochem. Biophys. Res. Commun. **453**:254–267.
25. **Webber MA**, **Piddock LJ V**. 2003. The importance of efflux pumps in bacterial antibiotic resistance. J. Antimicrob. Chemother. **51**:9–11.
26. **Piddock LJ V**. 2006. Multidrug - resistance efflux pumps — not just for resistance. Nat. Rev. Microbiol. **4**:629–636.
27. **Nesme J**, **Cécillon S**, **Delmont TO**, **Monier JM**, **Vogel TM**, **Simonet P**. 2014. Large-scale metagenomic-based study of antibiotic resistance in the environment. Curr. Biol. **24**:1096–1100.
28. **Miller R V.**, **Gammon K**, **Day MJ**. 2009. Antibiotic resistance among bacteria isolated from seawater and penguin fecal samples collected near Palmer Station, AntarcticaThis article is one of a selection of papers in the Special Issue on Polar and Alpine Microbiology. Can. J. Microbiol. **55**:37–45.
29. **Farias P**, **Santo CE**, **Branco R**, **Francisco R**, **Santos S**, **Hansen L**, **Sorensen S**, **Morais P V.** 2015. Natural hot spots for gain of multiple resistances: Arsenic and antibiotic resistances in heterotrophic, aerobic bacteria from marine hydrothermal vent fields. Appl. Environ. Microbiol. **81**:2534–2543.
30. **Hatha AAM**, **Neethu CS**, **Nikhil SM**, **Rahiman KMM**, **Krishnan KP**, **Saramma A V.** 2015. Relatively high antibiotic resistance among heterotrophic bacteria from arctic fjord sediments than water - Evidence towards better selection pressure in the fjord sediments. Polar Sci. **9**:382–388.
31. **Walsh C**. 2003. Antibiotics: Actions, Origins, ResistanceFirst. ASM Press, Washington D.C.

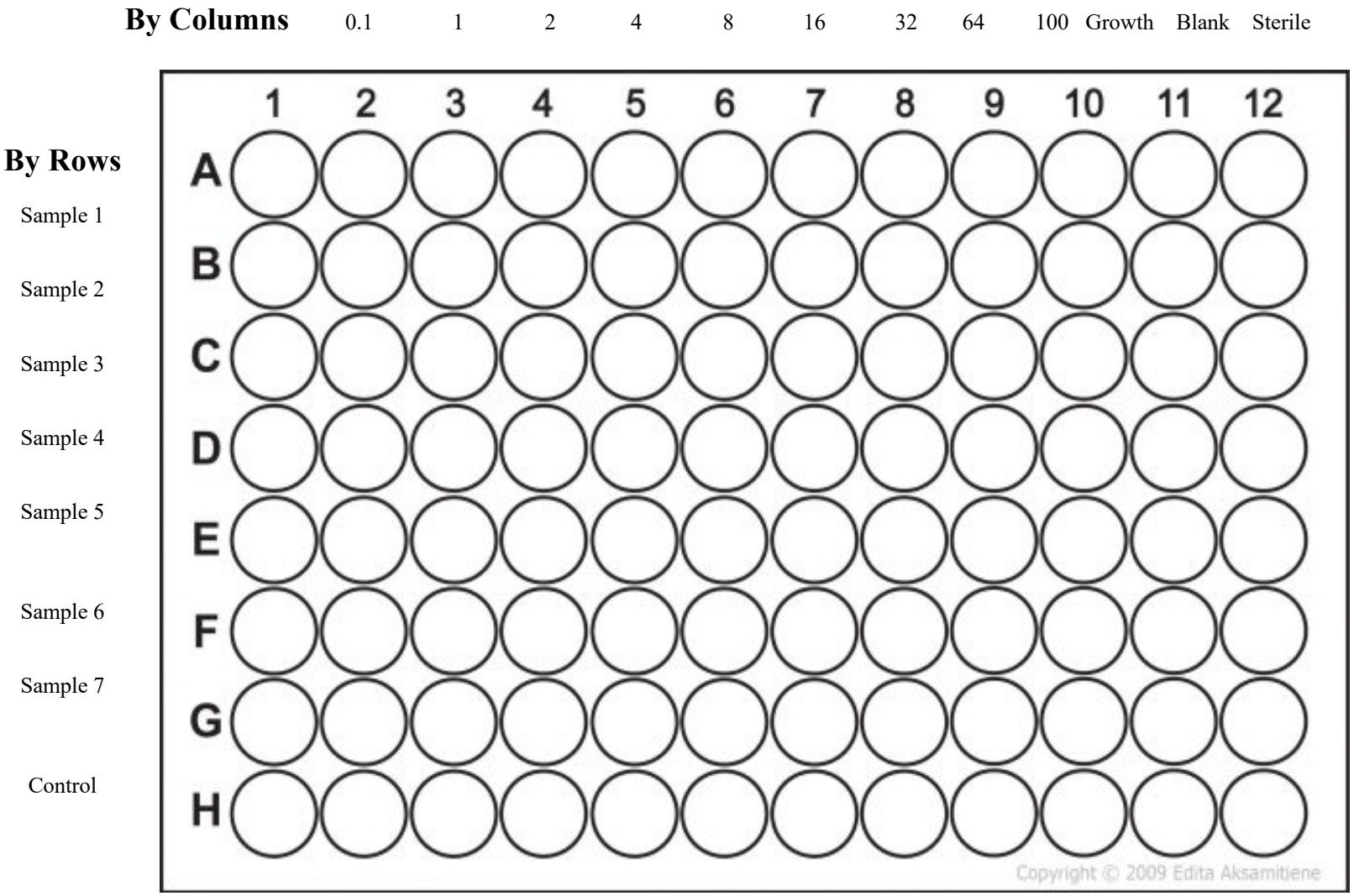


Figure 1. Minimum inhibitory concentration.
96 well plate setup for a plate testing a particular antibiotic. Growth refers to growth control, sterile refers to sterile control. 96 well plate image from: http://ideastocker.com/54-96-well-plate-template-gallery/96-well-plate-template-ideal-pics-plate-96-well-plate-template/

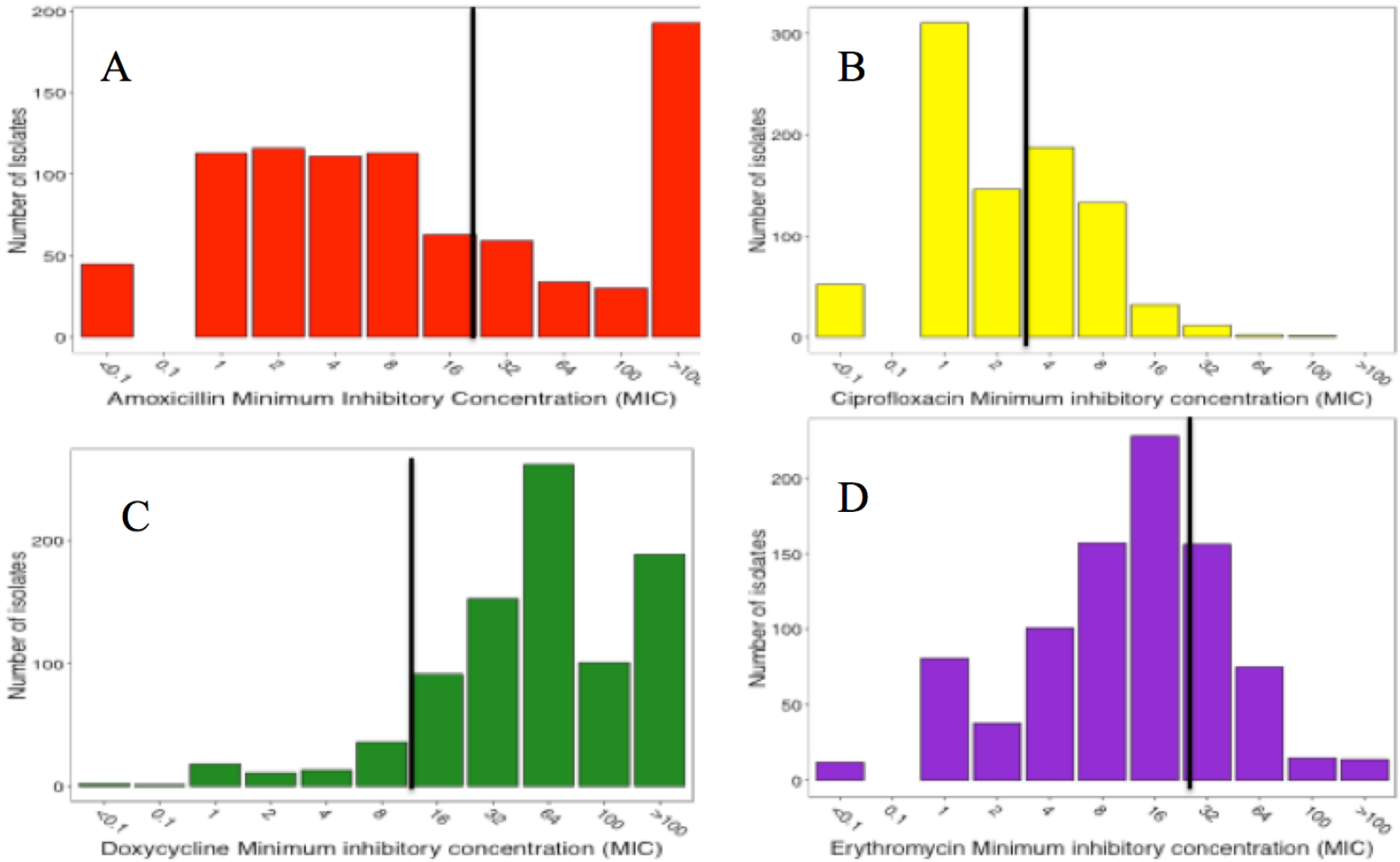
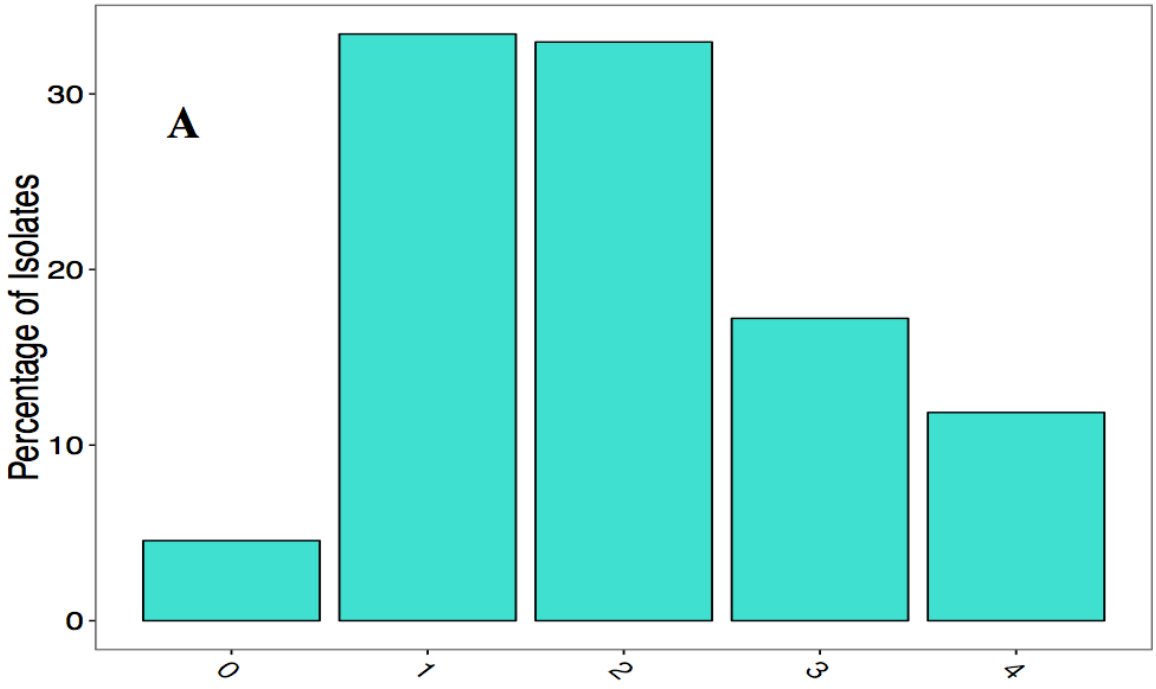


Figure 2. Minimum inhibitory concentration (MIC) values.
MIC in µg/ml for heterotrophic bacteria for a.) Amoxicillin, b.) Ciprofloxacin, c.) Doxycycline, and d.) Erythromycin. The black line indicates the breakpoint for resistance therefore, to the right of the black line indicates resistant isolates.


A
Percentage of Isolates
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20
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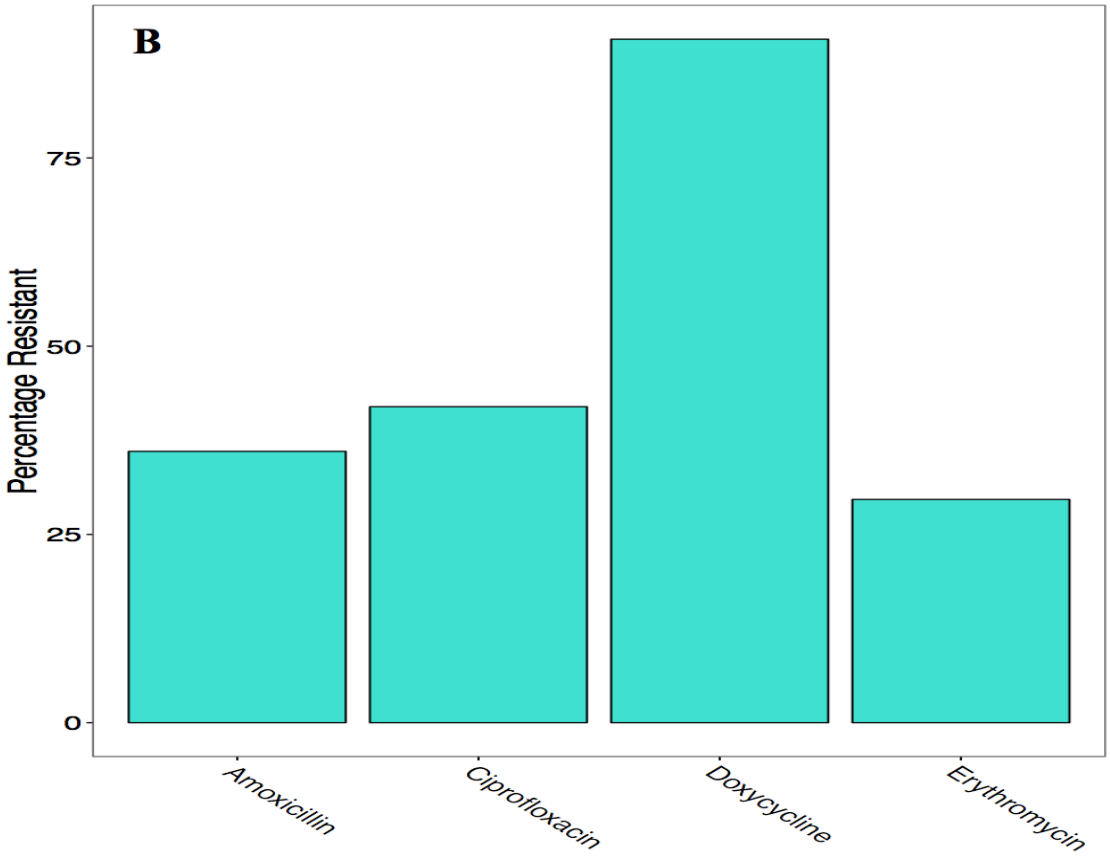
0
0
1
2
3
4


B
Percentage Resistant
75
50
25
0
Amoxicillin
Ciprofloxacin
Doxycycline
Erythromycin

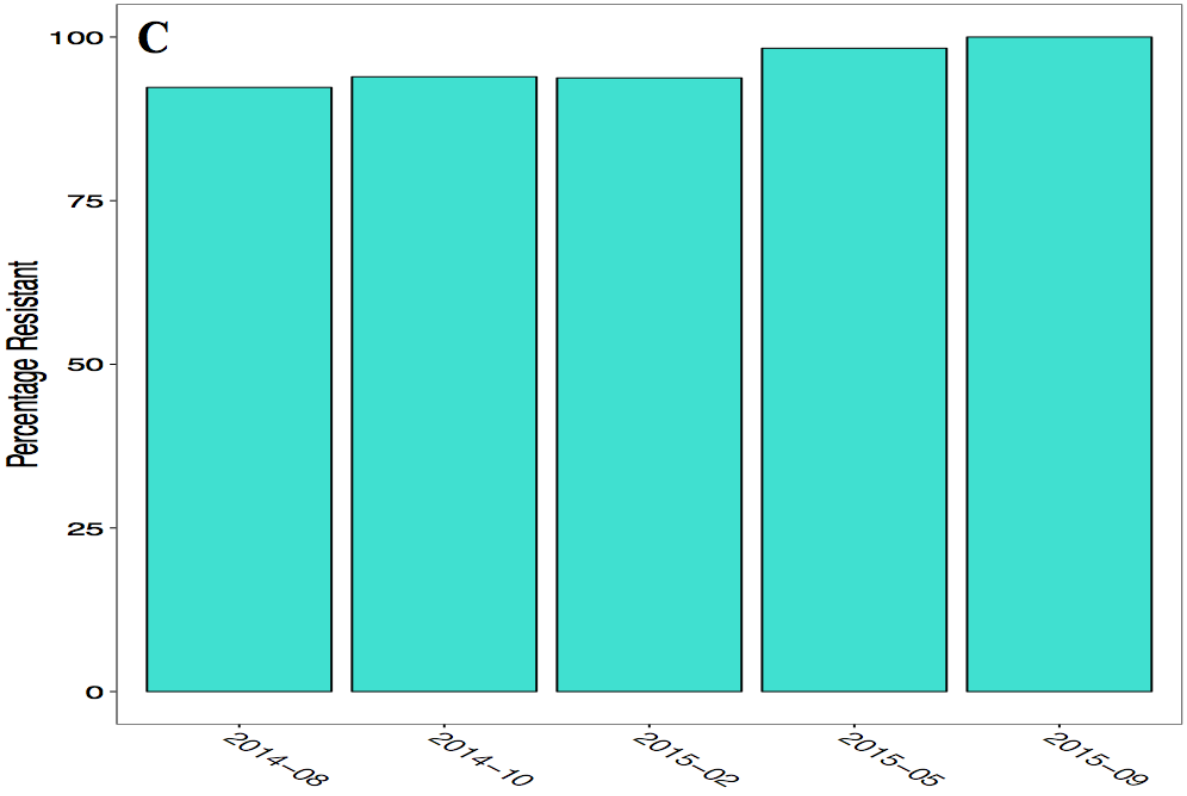


Figure 3: Resistance summary.
A) percentage of isolates resistant to 0,1, or more antibiotics. B) percentage of isolates resistant to each of the four tested antibiotics. C) percentage of isolates resistant to at least one antibiotic during each month.

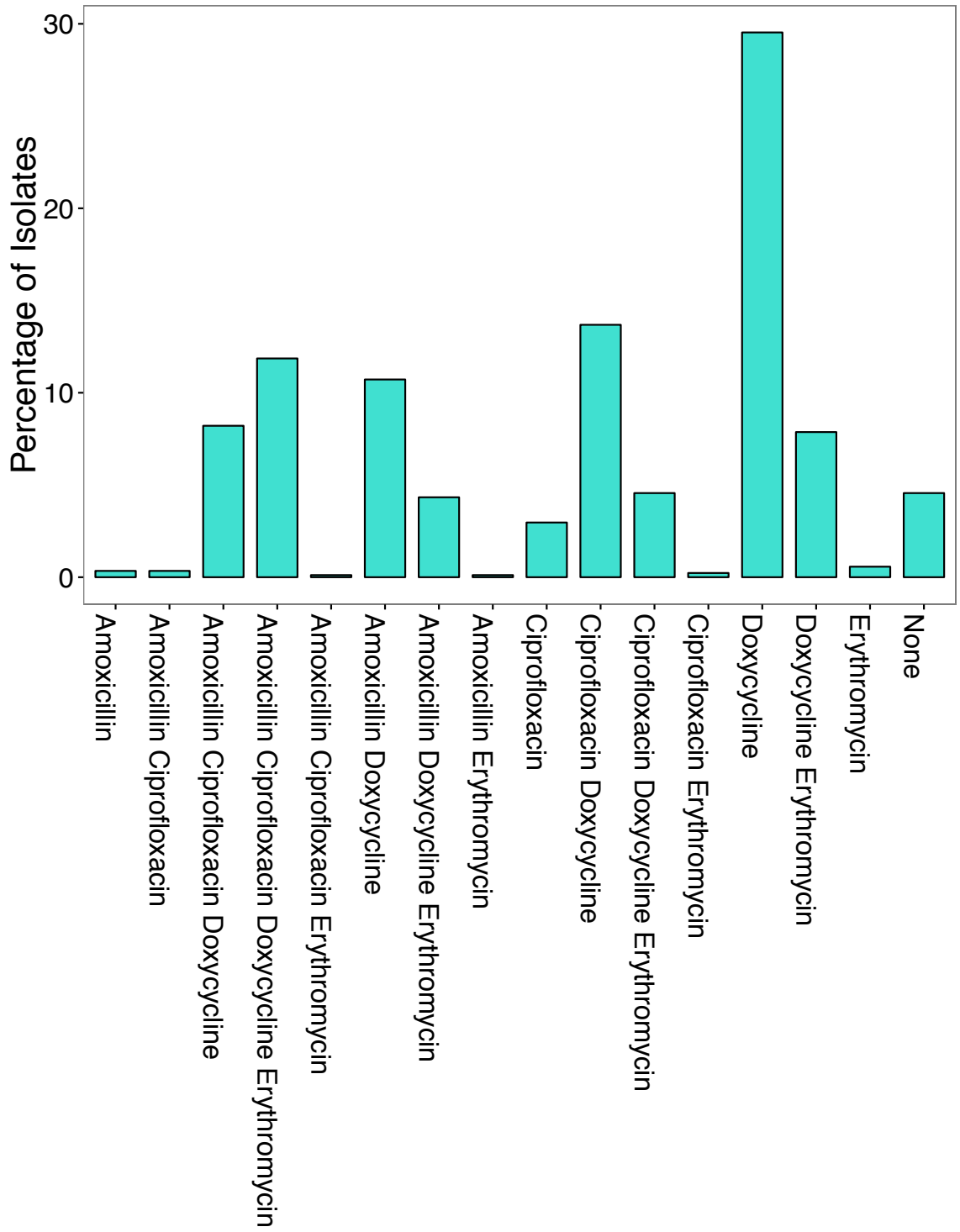


Figure 4. Percentage of resistant data illustrating prevalence of resistance categorization. Resistance categorization can include resistance to 0, 1, 2, 3, or 4 antibiotics.

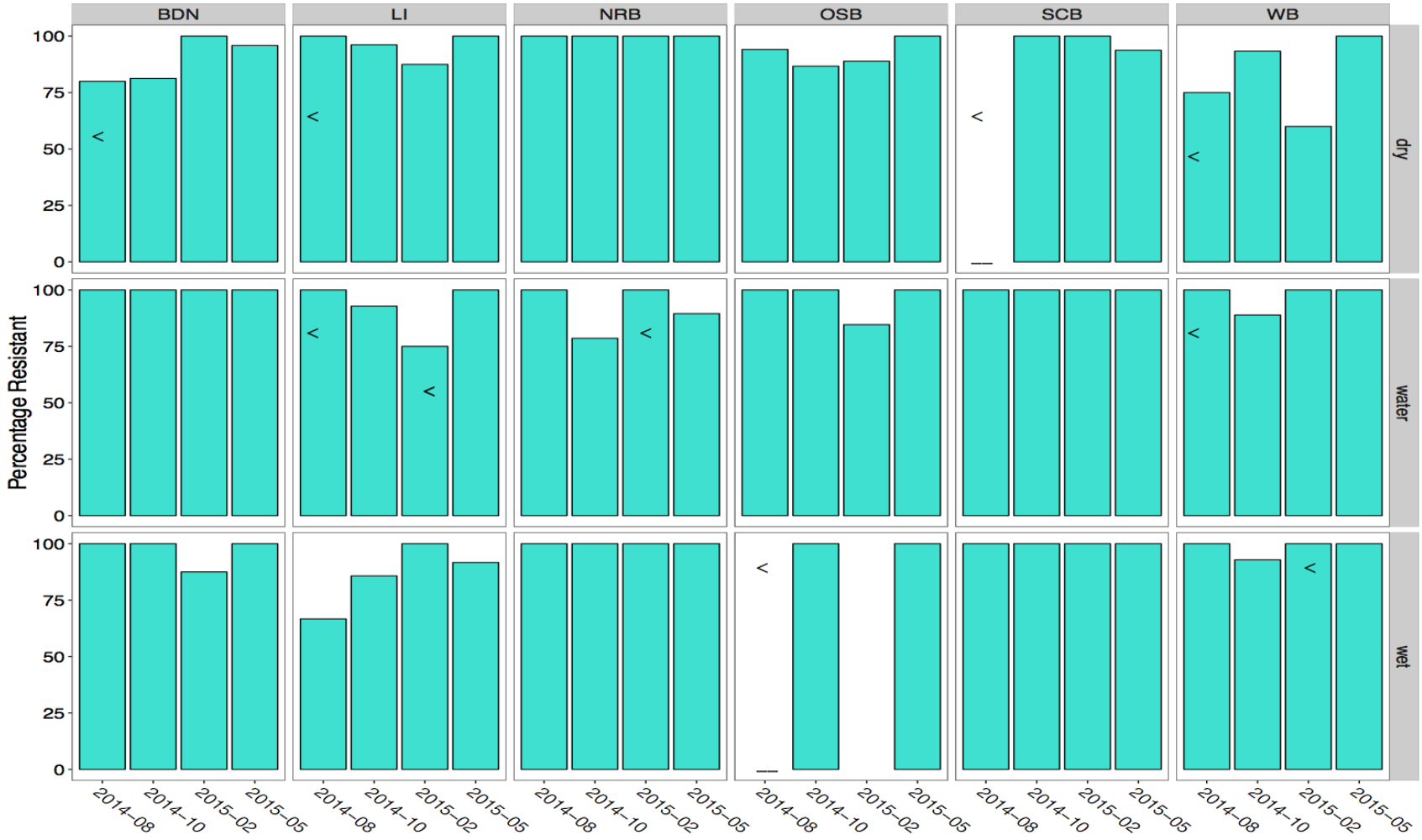


Figure 5. The proportion of isolates resistant to at least 1 antibiotic (y-axis) over time (axis).
Each graph indicates a different site and sample type. If all isolates from a sampling time point were sensitive to all antibiotics, a black dash is shown. An empty point indicates no samples from that sampling time were recovered for testing. Less than symbols indicate samples with 5 or fewer isolates.

Table 1. Antibiotics and resistance/sensitivity breakpoints used for isolates in this study.

Antibiotic	Antibiotic Class (37)	Mechanism (12, 38)	Activity (12)	Solvent for antibiotic (39)	Breakpoint for resistance	Organisms from the CLSI guidelines with this breakpoint (14, 40)
Amoxicillin	Penicillins (Beta-lactams)	Cell wall synthesis	Broad-spectrum	DMSO	32	Enterobacteriaceae
Ciprofloxacin	Quinolones	DNA gyrase	Broad spectrum	Water	4	*Entererobacteriaceae* *Pseudomonas aeruginosa* *Acinetobacter* Non-*Enterobactericeae* *Staphylococcus* *Enterococcus* *Abiotrophia/Granulicatella* *Aeromonas hydrophia* complex & *Plesiomonas shigelloides* *Bacillus* spp (other than B. anthracis) *Campylobacter jejuni/coli* *Corynebaterium* HACEK group[1] *Vibrio* spp (not *V. cholerae*)
Doxycycline	Tetracycline	30S ribosomal subunit	Broad spectrum	Water	16	Enterobacteriaceae *Acinetobacter* Non-*Enterobactericeae* *Staphylococcus* Enterococcus *Corynebacterium*

[1] HACEK: The Aphrophilus cluster of the Genus *Haemophilus* (i.e. *H. aphrophilus*, *H. paraphrophilus*, *H. segnis*), *Actinobacillus actinomycetemcomitans*, *Cardiobacterium* spp., *Eilenella corrodens*, and *Kingella* spp.)

Erythromycin	Macrolides	50S ribosomal subunit	Broad-spectrum	Water	32	*Campylobacter jejuni/coli*

Table 2. Bacterial controls used for MIC antibiotic resistance testing.

Control Number	Control Name	Antibiotic	MIC	Acceptable MIC values
2048	EI 90215 wet 2	Amoxicillin	>100	100, >100
1851	SCB 52215 wet H6	Ciprofloxacin	4	2-8
2048	EI 90215 wet 2	Doxycycline	>100	100, >100
516	OSB 81214 WH6	Erythromycin	8	4-16

Table 3. Isolate recovery.
Total number of isolates recovered and tested along with the percentage of isolates completed.

Sample	Total Isolates (number)	Tested Isolates (number)	Completed (%)
BDN 2014-08 dry	5	5	100
BDN 2014-08 water	7	6	85.7
BDN 2014-08 wet	6	6	100
BDN 2014-10 dry	16	16	100
BDN 2014-10 water	16	14	87.5
BDN 2014-10 wet	11	9	81.8
BDN 2015-02 dry	13	11	84.6
BDN 2015-02 water	7	7	100
BDN 2015-02 wet	8	8	100
BDN 2015-05 dry	24	24	100
BDN 2015-05 water	12	12	100
BDN 2015-05 wet	25	18	72
EI 2015-09 dry	23	23	100
EI 2015-09 water	12	12	100
EI 2015-09 wet	14	13	92.9
LI 2014-08 dry	3	2	66.7
LI 2014-08 water	3	3	100
LI 2014-08 wet	6	6	100
LI 2014-10 dry	26	26	100
LI 2014-10 water	15	14	93.3
LI 2014-10 wet	16	14	87.5
LI 2015-02 dry	8	8	100
LI 2015-02 water	7	4	57.1
LI 2015-02 wet	12	11	91.7
LI 2015-05 dry	17	17	100
LI 2015-05 water	12	10	83.3
LI 2015-05 wet	13	12	92.3
NRB 2014-08 dry	21	19	90.5
NRB 2014-08 water	5	5	100
NRB 2014-08 wet	12	12	100
NRB 2014-10 dry	8	7	87.5
NRB 2014-10 water	14	14	100
NRB 2014-10 wet	16	11	68.75
NRB 2015-02 dry	14	13	92.9
NRB 2015-02 water	3	3	100
NRB 2015-02 wet	8	8	100

NRB 2015-05 dry	21	21	100
NRB 2015-05 water	19	19	100
NRB 2015-05 wet	21	21	100
OSB 2014-08 dry	19	17	89.5
OSB 2014-08 water	9	8	88.9
OSB 2014-08 wet	4	2	50
OSB 2014-10 dry	19	15	78.9
OSB 2014-10 water	19	18	94.7
OSB 2014-10 wet	13	12	92.3
OSB 2015-02 dry	9	9	100
OSB 2015-02 water	14	13	92.9
OSB 2015-05 dry	16	14	87.5
OSB 2015-05 water	19	18	94.7
OSB 2015-05 wet	12	11	91.7
SCB 2014-08 dry	3	3	100
SCB 2014-08 water	7	7	100
SCB 2014-08 wet	8	6	75
SCB 2014-10 dry	18	17	94.4
SCB 2014-10 water	7	7	100
SCB 2014-10 wet	16	15	93.75
SCB 2015-02 dry	19	18	94.7
SCB 2015-02 water	13	11	84.6
SCB 2015-02 wet	15	14	93.3
SCB 2015-05 dry	17	16	94.1
SCB 2015-05 water	14	14	100
SCB 2015-05 wet	19	18	94.7
WB 2014-08 dry	6	4	66.7
WB 2014-08 water	5	5	100
WB 2014-08 wet	15	14	93.3
WB 2014-10 dry	17	15	88.2
WB 2014-10 water	14	9	64.3
WB 2014-10 wet	16	14	87.5
WB 2015-02 dry	10	10	100
WB 2015-02 water	11	10	90.9
WB 2015-02 wet	2	2	100
WB 2015-05 dry	18	17	94.4
WB 2015-05 water	12	12	100
WB 2015-05 wet	18	18	100

Table 4. Results from permutational multivariate analysis of variance (PERMANOVA) in Primer.
These data do not include the Elizabeth Island data because this site was only sampled on one occasion. Bolded and italicized factors indicate significant results. Italicized only indicates factors that were pooled.

Dataset	Question	Factors tested (factor type)	Results (p value, variance percentage)
Isolate Matrix	Does human impact affect resistance patterns?	Human Impact (fixed) Month (fixed) Sample Location (random) (nested in Human Impact) Sample Type (random) (nested in Sample Location)	*Impact (0.803, -2.0073)* ***Month (0.007, 6.8562)*** Location (0.425, 1.0013) Impact x. Month (0.831, -3.7642) ***Type (0.001, 4.5923)*** Location x Month (0.087, 4.955) ***Month x. Type (0.001, 8.6691)*** Residual variance: 19.121% Pooled Impact ***Month (0.005, 6.8562)*** Impact x. Month (0.839, -3.7642) ***Type (0.001, 4.5923)*** Month x. location (0.086, 4.955) Month x. type (0.001, 8.6691) Residual variance: 19.121%
	Does watershed affect resistance patterns?	Watershed (fixed) Month (fixed) Sample Location (random) (nested in Watershed) Sample Type (random) (nested in Sample Location)	Watershed (0.201, 3.0086) ***Month (0.016, 7.1149)*** *Location (0.905, -2.7525)* Watershed x. Month (0.882, -5.0254) ***Type (0.001, 4.5923)*** Location x. Month (0.065, 5.4856) ***Month x. Type (0.001, 8.6691)*** Residual variance: 19.121% Pooled Location Watershed (0.163, 2.2547) ***Month (0.024, 7.1149)***

			Watershed x. month (0.88, -5.0254) Month x. location (0.043, 5.4856) ***Month x. type (0.001, 8.6691)*** Residual variance: 19.121
RI Count Matrix	Does human impact affect resistance prevalence?	Human Impact (fixed) Month (fixed) Sample Location (random) (nested in Human Impact) Sample Type (random) (nested in Sample Location)	*Impact (0.952, -1.4884)* ***Month (0.01, 7.1374)*** *Location (0.677, -2.5461)* Impact x. Month (0.403, 1.8553) *Sample Type (0.606, -2.635)* Month x. Type (0.234, 4.5832) Residual variance: 16.562% Pooled Sample type *Impact (0.934, -1.3807)* **Month (0.006, 7.2326)** *Location (0.815, -3.0815)* Impact x month (0.42, 2.2516) Month x. location (0.182, 4.6667) Residual variance: 16.351% Pooled Location *Impact (0.726, -2.1694)* ***Month (0.005, 7.1374)*** Impact x. Month (0.426, 1.8553) Month x. Location (0.241, 4.5832) Residual variance: 16.562 Pooled Impact ***Month (0.011, 7.1374)*** Impact x. Month (0.437, 1.8553) *Sample Type (0.648, -2.635)* Month x. Location (0.208, 4.5832) Residual variance: 16.562%
	Does watershed affect	Watershed (fixed)	Watershed (0.165, 4.1049)

resistance prevalence?	Month (fixed) Sample Location (random) (nested in Watershed) Sample Type (random) (nested in Sample Location)	***Month (0.024, 7.9884)*** *Location (0.949, -3.7711* Watershed x. Month (0.236,5.4489) *Sample type (0.625, -2.635)* Month x. Location (0.303, 3.7651) Residual variance: 16.562% Pooled Sample Type Watershed (0.166, 4.2208) ***Month (0.021, 8.0297)*** *Location (0.993, -4.1455)* Watershed x. month (0.212, 5.5031) Month x. location (0.271, 3.9999) Residual variance: 16.351% Pooled Sample Location Watershed (0.105, 3.2492) Month (0.028, 7.9884) Watershed x. month (0.228, 5.4489) Month x. location (0.288, 3.7651) Residual variance: 16.562%

Table 5. Estimated amount of resistant bacteria ingested.
Ingested bacteria by individuals participating in a given recreational activity by site for water samples collected in October 2014. The amount of water ingested in each of these activities comes from Leonard (41). Amount of resistant bacteria includes the estimates for bacteria resistant to 1,2, 3, or 4 antibiotics.

Recreational activity	Amount of water (ml)	BDN 2014-10 water	LI 2014-10 water	NRB 2014-10 water	OSB 2014-10 water	SCB 2014-10 water	WB 2014-10 water
Swimming-children	37	1.6×10^5	6.9×10^4	1.6×10^5	4.8×10^5	7.3×10^5	18×10^5
Swimming-adults	16	7.1×10^4	3.0×10^4	6.9×10^4	2.1×10^5	3.2×10^5	7.7×10^4
Boating	3.7	1.6×10^4	6.9×10^3	1.6×10^4	4.8×10^4	7.3×10^4	1.8×10^4
Rowing	3.5	1.6×10^4	6.6×10^3	1.5×10^4	4.6×10^4	6.9×10^4	1.7×10^4
Canoeing	3.9	1.7×10^4	7.3×10^3	1.7×10^4	5.1×10^4	7.7×10^4	1.9×10^4
Kayaking	3.8	1.7×10^4	7.1×10^3	1.6×10^4	5.0×10^4	7.5×10^4	1.8×10^4
Fishing	3.6	1.6×10^4	6.8×10^3	1.6×10^4	4.7×10^4	7.1×10^4	1.7×10^4
Wading/splashing	3.7	1.6×10^4	6.9×10^3	1.6×10^4	4.8×10^4	7.3×10^4	1.8×10^4
Diving	9.9	4.4×10^4	1.9×10^4	4.3×10^4	1.3×10^5	2.0×10^5	4.8×10^4

Table 6. Estimated amount of resistant bacteria filtered per hour by a variety of shellfish.
The low and high estimates are based on a range of water filtration capabilities for the organisms. Amount of resistant bacteria includes the estimates for bacteria resistant to 1,2,3, or 4 antibiotics.

Shellfish		**Filtration rate**		**BDN 2014-10 water**	**LI 2014-10 water**	**NRB 2014-10 water**	**OSB 2014-10 water**	**SCB 2014-10 water**	**WB 2014-10 water**
		L/h	ml/h						
Crassostrea virginica	Low filtration rate	2.04	2040	9.0×10^6	3.8×10^6	8.8×10^6	2.7×10^7	4.0×10^7	9.8×10^6
	High filtration rate	27	27000	1.2×10^8	5.1×10^7	1.2×10^8	3.5×10^8	5.3×10^8	1.3×10^8
Mercenaria mercenaria	Low filtration rate	0.3	300	1.3×10^6	5.6×10^5	1.3×10^6	3.9×10^6	5.9E x 10^6	1.4×10^6
	High filtration rate	3.6	3600	1.6×10^7	6.8×10^6	1.6×10^7	4.7×10^7	7.1×10^7	1.7×10^7
Mya arenaria	Low filtration rate	1.7	1700	7.5×10^6	3.2×10^6	7.3×10^6	2.2×10^7	3.4×10^7	8.2×10^6
	High filtration rate	7.4	7400	3.3×10^7	1.4×10^7	3.2×10^7	9.7×10^7	1.5×10^8	3.6×10^7

CHAPTER 5 Diversity of antibiotic resistance associated genes and elements in coastal marine metagenomes

Introduction

In recent years, a greater understanding of the environment as a reservoir for antibiotic resistance has emerged. Within this idea are two important subtopics. One is that anthropogenic use of antibiotics can allow antibiotics and antibiotic resistant bacteria to enter into natural environments (1). The second is often forgotten, that resistance is a natural process that has existed long before humans ever started utilizing antibiotics for their own needs (2, 3). This idea of the innerconnectedness between humans and the natural environment, along with animals, is often called the "One Health" concept and views that these areas should be studied as one connected system instead of separate spheres that do not overlap.

The ocean is both a natural environment, containing native bacteria with antibiotic resistance genes (ARGs) and also often a dumping ground for anthropogenic waste, including industrial and wastewater pollution. Industrial pollution often includes metals, which are believed to allow for co-selection to antibiotic resistance (4, 5). One study from 2000 showed that a stream affected by industrial pollution had higher levels of resistance compared to the reference stream (6). Wastewater pollution represents a more direct antibiotic impact from humans as up to 90% of these drugs are excreted in waste products (7) and most wastewater facilities are not able to remove these chemicals due to the high cost and effort necessary. Wastewater treatment plants are also known to be hotspots for antibiotic resistance transfer (8) and have increased resistance downstream of a given wastewater plant compared to upstream (9). These results indicate that higher levels of resistance may be present in coastal marine environments that have been affected by industrial and/or wastewater pollution.

Sequencing technology has transformed biology and allowed for a greater understanding of the microbial world that otherwise can be invisible and difficult to culture. Metagenomics, or the sequencing of DNA directly from the environment, is critical in the understudied marine environment. Metagenomics in the ocean has allowed discoveries of novel enzymes (10, 11) to nitrogen fixing microbes in the surface ocean (12) to strategies of marine viruses (13). Even more discoveries can be anticipated as sequencing becomes less expensive and more accessible, allowing for experiments that once seemed unattainable to become reality.

With respect to antibiotic resistance (AR), metagenomics facilitates the detection of both known and novel resistance genes from environmental samples, where it is estimated that only ~1% of the bacteria are successfully cultured (14–16). The caveat for this method is that metagenomics only allows for assessment of AR potential because it is unknown if resistance is being actively expressed in a particular organism or when that occurred. Port et al 2012 used early sequencing technology to elucidate 18 antibiotic resistance determinants in coastal samples (17) and an expanded study illustrated that site and season were important in determining the amount of resistance determinants (18). Nesme et al. 2014 completed a meta-analysis showing that resistance was present in ocean samples and in fact, every environmental sample surveyed (19). In a more recent study, Uyaguari-Diaz et al found fewer types and copies of ARGs in low impact freshwater environmental samples, while more impacted samples had greater abundance and diversity of resistance (7). Hatsoy and Martiny studied antibiotic resistance genes in the ocean through the use of functional metagenomics, cloning DNA from an environmental

sample into *E.coli* and then selecting and sequencing isolates demonstrating resistance to specific antibiotics (20). They found a variety of known and unknown resistance genes, providing evidence of the ocean not only as a reservoir for resistance to antibiotics but as a potential source for new resistance.

This study seeks to better describe the diversity and distribution of antibiotic resistance genes in coastal marine environments with different environmental conditions. Samples were collected from sites with industrial or wastewater pollution impacts, as well as unimpacted sites deemed as reference sites, to compare the effect of these different conditions on ARG diversity and abundance. The diversity and abundance of ARGs are examined by using metagenomic sequence analysis. Samples of sediment and water not only allow an understanding of what resistance genes are present, but can also serve as a proxy for time. Understanding the diversity and prevalence of ARG facilitates better interpretation of humans' interaction with, and influence on, aquatic systems and how it may result in the environment playing a role in the persistence and spread of antibiotic resistance. Environments with more anthropogenic environmental impact likely have overall a greater abundance of resistance genes. Previous research showed lower relative abundance and diversity in unimpacted samples (7). Therefore the less anthropogenically impacted environmental samples in this study could have fewer ARGs and reduced diversity compared to more impacted environments.

METHODS

Study Sites

Samples were collected from ten sites: Charles River, New Bedford Harbor, Plymouth, West Falmouth Harbor, Little Island, Race Point Beach, Falmouth Wastewater Treatment Influent and Effluent, Boston Harbor, and Boston Deer Island Outfall. More details about the sites are available in Table 1.

Field sampling collections

For samples with both water and sediment collected (Charles River, New Bedford Harbor, Plymouth, West Falmouth Harbor, Little Island, Race Point Beach), three one liter autoclaved acid washed bottles were used to collect water at roughly 30-40 cm depth. Three sterile 50 ml centrifuge tubes were used to collect sediment underlying the water column at the same point. For the Falmouth wastewater treatment plant samples, water samples were collected by pulling up water from influent or effluent tanks and then pouring into three one-liter autoclaved acid washed bottles. For these sites, environmental measurements were collected (Table 2).

Water from Boston Harbor and Deer Island outfall was collected in 50 liter carboys. Triplicate samples were collected from all sites except Boston Harbor and the Deer Island outfall. All samples other than the Boston Harbor and Deer Island site were transported in coolers back to the laboratory and processed immediately. The Boston Harbor and Deer Island carboys were put at 4°C upon arrival back in the laboratory on April 26, 2016 and then were filtered on April 29, 2016.

Laboratory sample filtration

Water samples other than Boston Harbor and Outfall samples were serially filtered through an acid-washed 5 um mesh filter and then onto an autoclaved 47 mm 0.22 micron Durapore filter (Millipore). Amounts of water filtered are given in Table 1 in detail. For the Boston Harbor and outfall samples, about three liters for both were filtered through sequential filters of an 80 micron filter to 35 micron to 0.22 micron filter.

For sediment samples, 5 grams of sediment was measured and added to 15 ml of sterile 1x phosphate buffered saline (PBS). The sample was lightly shaken (15 times back and forth) to allow cells to be dislodged from sediment. The sample was allowed to sit for a few minutes for settling of large sediment particles, then the PBS was filtered through 5 micron mesh onto 0.22 micron filter (Durapore; Millipore). After filtration, all samples were stored at -80 °C until extraction.

DNA Extraction

For sediment and water DNA extraction from filters, the Genomic-tip 20/G process was used (Qiagen). The lysis protocol was followed (21) with modifications. To lyse cells, each filter was rinsed with Qiagen Buffer B1 and then 5 µl of 250 U/µl Ready-Lyse Lysozyme (Epicentre Illumina) was added for 30 minutes at room temperature. To remove RNA and protein, 2 µl RNAse A (Qiagen) along with 45 µl of protease (Qiagen) was added and incubated for thirty minutes at 37 °C. For some samples, two filters were used to increase DNA yield and in these cases, doubled amounts of reagents were used for the additional increase of Buffer B1. The Qiagen protocol for Bacteria was continued at Step 6, with the addition of Buffer B2 and the DNA extraction process was followed for 20/G tips (Qiagen).

Metagenomic sequencing

DNA samples were sent to Georgia Genomics and Bioinformatics Core for library preparation and sequencing. Library preparation used the Kapa Hyper Kit (KK8504) with fragmentation and dual SPRI size selection following the Illumina TruSeq protocol. Sequencing occurred on an Illumina NextSeq machine with 150 base pair paired end sequencing. Upon receipt of the sequence data, it was determined that four samples needed to be resequenced due to low read numbers. These samples were LI Water 1, LI Water 2, NBH Water 3, and the Outfall Water.

Bioinformatic analysis

Bioinformatic analysis utilized MetaStorm (22). MetaStorm is an online server that allows for a user-friendly metagenomic pipeline that can be customized to the users needs. The general data flow is illustrated in Figure 1. Two different pipelines were utilized in the data analysis: an assembly based pipeline and a read matching pipeline. The assembly based pipeline was used for the identification of antibiotic resistance related genes whereas the read matching was used for bacterial taxonomic identification and diversity. MetaStorm uses a variety of software for data processing: quality control (Trimmomatic), assembly (IDBA-UD), gene prediction (PRODIGAL), taxonomy annotation (BLAST, DIAMOND, GreenGenes for 16S rRNA for the read matching pipeline) and functional annotation (DIAMOND for the assembly pipeline). A CLAssification of Mobile genetic Elements (ACLAME), Antibiotic Resistance Database (ARDB), Comprehensive Antibiotic Resistance Database (CARD), and Antibacterial Biocide and Metal Resistance Genes Database (BacMet) databases were used for annotation of the assembly pipeline data. ACLAME is a database full of mobile genetic elements and is available at

http://aclame.ulb.ac.be/. ARDB is a non-currently updated database of antibiotic resistance genes which is available at https://ardb.cbcb.umd.edu/. CARD is an updated database of antibiotic resistance genes and can be found at https://card.mcmaster.ca/about. BacMet includes biocide and metal resistance genes and is available at http://bacmet.biomedicine.gu.se/. To account for different total read recovery for each sample, gene abundances were normalized to RPKM for the read matching (taxonomy) analysis and to 16S read abundance for the assembly (ARG) analyses.

Statistical analysis

Statistical analysis was completed using Primer version 7 (23). Permutational multivariate analysis of data (PERMANOVA) was utilized to compare gene diversity and prevalence. PERMANOVAs were completed on gene abundance data, metagenomic sample matrices, and efflux pump data. Gene abundance data was analyzed for significant differences in the normalized gene abundance between samples using a nested PERMANOVA with sample type nested in site nested in impact type. This test was completed for data from each database: ACLAME, ARDB, CARD, BacMet, and GreenGenes.

Eight different types of metagenomic sample datasets were created to address a variety of questions. The datasets are: all samples, all water samples, water samples without effluent and influent, six main sites that included sediment and water, wastewater water and sediment, wastewater (water only), influent/effluent, and the WFH gradient (effluent, influent, WFH Water). For each of these eight datasets, a matrix was created for each of six databases: ARDB gene, ARDB antibiotic, BacMet, CARD gene, CARD

antibiotic, and GreenGenes. In addition, similarity percentages (SIMPER) were run on the top ten genes or group for each of five databases (ACLAME, ARDB, CARD, BacMet, and GreenGenes) to examine what similarities and differences were present between sites and between sample types.

RESULTS

Sampling sites

10 different sites were sampled during this study in the summer/autumn of 2016. Six sites were sampled for both sediment and water whereas the other four sites were sampled solely for water (Table 1). Environmental characteristics of the sampling sites varied with conductivity, salinity, turbidity, dissolved oxygen and temperature (Table 2). Barometric pressure and previous rainfall had less variability among the sites. Turbidity varied between the sites with influent having the highest value, while the industrial sites of New Bedford Harbor and Charles River have the next highest amounts. Both of these sampling points can be considered having estuarine or riverine influences (the New Bedford Harbor site is on the Acushnet River), which may increase the sediment load as the flow moves along and less flushing occurs in these systems. Salinity also varied among sites, with New Bedford Harbor, West Falmouth Harbor, Little Island, and Provincetown having the highest salinities whereas Charles River and Plymouth were very low, reflecting the freshwater influence on these coastal regions.

Metagenome assembly results

Metagenomes analyzed resulted in total raw reads from 9 to 25 million per sample (Table 3). The unassembled read matching pipeline (Figure 1) was used to determine taxonomic

structure of the samples with GreenGenes. Total reads that had hits to GreenGenes were less than one percent for all samples, and this was consistent with results from a TARA sample (Table 3). The assembled data showed that usually >30% of total reads were assembled for the coastal and TARA water samples (Table 4). Sediment samples often had fewer assembled reads (<10%), fewer scaffolds and ultimately fewer predicted genes compared to corresponding water samples (Table 4), but this was not always the case (e.g. NBH Sed 1&2 vs NBH Water 1&2). The number of predicted genes from all samples ranged from 5,959 (Ptown Sed 3) to 623,044 genes (NBH Sed 1). Using the assembled pipeline, four different databases were utilized to identify genes associated with antibiotic resistance (ARDB, CARD), metal resistance (BacMet), and mobile genetic elements (ACLAME). Overall, antibiotic resistance associated genes comprised <10% of the total genes in a sample (Table 5). ACLAME had the most (0.02 to 7%) followed by BacMet (0 to 0.4%), CARD (0 to 0.29%), and ARDB (0 to 0.16%).

Top database hits

Plasmids (ACLAME) were the most prevalent of the AR associated elements within the metagenomic data. Seven of the top twenty most abundant plasmid types have oxidoreductase activity while three are ATP-binding cassettes (Table 6). The remaining groups have diverse descriptions and mechanisms; two of particular interest are plasmid 2 (associated with toxicity via the Type III secretion pathway) and plasmid 74 (carries insertion element IS407). For the ARDB database, the top twenty genes contain a variety of resistance mechanisms that impact different antibiotic groups (Table 7). Efflux related genes are in the majority with twelve out of twenty genes related to this process. The

CARD top 20 list has similar diversity to the ARDB database (Table 8). In addition, eleven out of twenty genes for the CARD database were related to efflux. BacA, dfrB6, macB, and tet34 are the four genes that are included on both the CARD and ARDB top 20 lists. The BacMet database illustrated a variety of metal resistance associated genes, with the category "other" as the top amount (Table 9). This category includes genes that have multiple annotations or other compounds. GreenGenes top 20 genera includes several "unknown bacteria" in addition to taxa commonly found in marine samples (Table 10). Figure 7 shows the larger expanse of prokaryotic diversity, instead of the top 20 samples.

PERMANOVA tests

Gene abundance

A nested PERMANOVA test was run on a matrix for each database (ACLAME, ARDB, BacMet, CARD, and GreenGenes) containing gene abundance values for each sample. For all databases, sample type (sediment vs water) was significant (Table 11). Significance of sample type remained even if the factor of impact was pooled in the analysis (Table 11). Figure 2 illustrates that although sediment tended to have higher relative recovery abundances than water from the same site, the pattern did vary.

Analysis of all sample matrix

A matrix of all samples was created for each database to evaluate the diversity between samples. In addition, separate matrices were created for the antibiotic resistance databases ARDB and CARD for antibiotic group. Each database condensed all ARG into broader antibiotic groups (e.g. penicillin, tetracyclines) and then these matrices were tested as well. A nested PERMANOVA tested the significance of three factors: impact type

(wastewater, Boston wastewater, industrial, reference), site, and sample type (sediment or water) (Table 12, Column 2, red). For ARDB genes and antibiotic, CARD genes and antibiotic, and GreenGenes, sample type was significant at a p value of 0.001. The amount of variance sample type explained changed between databases- ARDB genes (25.406), ARDB-antibiotic (25.44), CARD-antibiotic (23.284), CARD-gene (25.505), GreenGenes (22.031). For BacMet, impact and sample type were not significant after the site factor was pooled.

All water samples matrix

Matrices of all water samples were created and were tested by PERMANOVA using a site nested in impact type (Table 12, Column 3, orange). Site was significant for all of the database results; ARDB genes (p = 0.001, 30.572), ARDB- antibiotic (p=0.002, 23.587), BacMet (p=0.007, 11.718), CARD-antibiotic (0.001, 31.16), CARD-gene (p=0.001, 33.587), and GreenGenes (p=0.001, 30.155). Figure 3 shows an nMDS plot using the ARDB gene data to illustrate the groupings by site. Some samples are extremely close together such as Influent and Effluent while others, like Provincetown, are more variable.

A water sample matrix without the effluent and influent samples was run (Table 12, Column 4, yellow). Site remained significant for CARD- gene (p=0.005, 17.868) and GreenGenes (0.001, 22.291) while it was not for ARDB genes, ARDB antibiotic, BacMet, and CARD- antibiotic. Figure 4 uses the GreenGenes matrix to illustrate that variability between site groups is larger than variability within groups for most samples.

Sediment and water sites only

Matrices of the six sites for which sediment and water were collected (CR, LI, NBH,

Plymouth, Provincetown, WFH) tested using PERMANOVA with sample type nested in site nested in impact type (Table 12, Column 5, green). As for the all sample analysis, type was significant, although in this case it was significant for all databases other than CARD-gene. For each database, the significant results for type were: ARDB genes (0.002, 23.734), ARDB antibiotic (0.001, 24.338), BacMet (0.001, 17.695), CARD antibiotic (0.001, 22.234) and GreenGenes (0.001, 28.524).

Wastewater samples only

All wastewater samples, including sediment and water samples, were tested by a PERMANOVA with type nested in site nested in impact type (Table 12, Column 6, light blue). Sample type was significant for all of the datasets; ARDB genes (0.001, 32.448), ARDB- antibiotic (0.001, 31.233), BacMet (0.001, 21.503), CARD-antibiotic (0.011, 20.412) CARD-gene (0.002, 24.904), and GreenGenes (0.001, 26.497). Impact type was also significant for BacMet (0.023, 19.356). When site was pooled for BacMet, due to its p value and negative significance, the significance of sample type was not able to be tested. Site was only significant for CARD- antibiotic (0.026, 27.141).

A matrix of wastewater water samples only was tested using site nested in impact type (Table 12, Column 7, dark blue). Site was again a significant factor with this setup for all datasets; ARDB genes (0.001, 38.585), ARDB antibiotic (0.001, 30.004), BacMet (0.004, 15.237), CARD antibiotic (0.001, 37.061), CARD-gene (0.001, 45.091), and GreenGenes (0.039, 22.099). Figure 5 Panels A and B show the wastewater water sample nMDS results and illustrates that effluent and influent form distinct groups and cluster away from the other samples. In addition, taxonomy differences were also shown within

these samples, which may be related to the differences in resistance (Figure 5 Panel D). Influent and effluent were then tested for differences by site within PERMANOVA (Table 9, Column 8, purple), but site was not shown to be significant for any database.

WFH Gradient

The WFH Gradient samples (influent, effluent, WFH water) were tested for PERMANOVA by site (Table 12, Column 9, beige). Site was significant for all databases: ARDB genes (0.006, 47.04), ARDB antibiotic (0.005, 35.492), BacMet (0.003, 20.041), CARD-antibiotic (0.007, 45.932), CARD gene (0.006, 49.961), GreenGenes (0.004, 40.487). The sites samples cluster into are shown in Figure 6.

Antibiotic resistance results across methods: Little Island comparison

Little Island was a site studied in three chapters of this dissertation (3- *Vibrio*, 4-heterotroph, and in this chapter, in coastal metagenomes). Antibiotic resistance for each chapter is illustrated in Table 13. *Vibrio* isolates had higher amounts of resistance for amoxicillin (a penicillin), ciprofloxacin (a quinolone), and trimethoprim than for doxycycline (a tetracycline) and oxytetracyline (a tetracycline). Heterotrophs had higher resistance for doxycycline and reduced levels for amoxicillin, ciprofloxacin, and erythromycin (a macrolide). Within the coastal metagenomes, a diverse set of resistance genes was found. Macrolide, bacitracin, lincosamide/streptogramin_b/macrolide grouping, kasugamycin, and tetracycline were the most abundant, and genes related to the identified active resistances in the bacteria were also found.

Similarities between sites and sample type using SIMPER

Similarity percentage (SIMPER) tests, which discriminate dissimilarities between samples,

were performed on the top 10 genes or groupings over all samples for each database to determine the genes/taxa that characterized each site or sample type and contributed to the significant PERMANOVA results. Table 14 shows the SIMPER results by sample type while Table 15 reports on results of specific comparisons between sample types and sites. The plasmid (ACLAME database) and BacMet genes had the least variation in type abundances. Overall water vs sediment dissimilarity was 17% and 26% (Table 11), while the dissimilarity between different sites was < 22% and < 35%, for plasmids and BacMet respectively (Table 12). The greatest dissimilarity in the BacMet dataset was between influent and Deer Island outfall samples (36% dissimilarity; mercury, copper & zinc higher for influent; selenium & chromium higher for outfall). BacMet was the only dataset to show significance for sample impact, and with SIMPER the largest dissimilarity (28%) was between Boston wastewater (Outfall, Boston Harbor) and wastewater (effluent, influent, Plymouth, WFH), with Boston showing more chromium and selenium genes, while wastewater had more mercury, arsenic and copper genes.

ARDB and CARD results had the largest variability within samples (between replicates) with similarity values ranging from 14% up to 89% (Table 15). Sediment and water were more distinct here with dissimilarity values for ARDB and CARD of 58% and 47%, respectively (Table 14). The genes driving these differences in ARDB were AcrB for sediment and KsgA and mexW for water. In CARD, the genes were CRP for sediment and pmrE and qacH for water. Provincetown as a site had the lowest average similarity value (ARDB 19%; CARD 47%) reflecting the large differences observed in the location of replicate samples on MDS plots (Figure 3). In ARDB dataset site comparisons, those with

Provincetown tended to have the largest dissimilarity values (66%-80%), and were influenced by the increased abundance of tet34 and AcrB in the Provincetown samples relative to the others. A similar pattern was observed for CARD (38%-58%), with the genes CRP and msrB usually of higher abundance in Provincetown samples.

Although impact was not significant in most of the PERMANOVA results, it was possible to identify genes associated with the distinction of influent and effluent samples, as well as wastewater. In ARDB, effluent was distinguished from influent by larger amounts of dfrB6 and BacA, and lower amounts of RosA, while wastewater as an impact type tended to have higher mexW gene abundances. mtrA, qacH and rpoB genes from the CARD database were generally of greater abundance in effluent samples, while pmrE and CRP were more abundant in influent. Wastewater showed an increase in the effluent and influent genes, qacH and pmrE, in addition to msrB and dfrA3.

Within the GreenGenes dataset, there was little variability between most sample replicates, and often also between sediment and water from the same site (Table 14, 15). A notable exception was the Provincetown Total sample where the low similarity value indicated the water and sediment differences between these communities. GreenGenes results yielded the greatest variation in site dissimilarity values, ranging from 8% (Boston Harbor vs outfall) up to 75% (Provincetown vs influent). Due to the limited number of taxa used, the same species were generally found in each comparison, while the abundances were different. An exception to this observation was that the presence and abundance of *Arcobacter* did characterize wastewater impact from others.

Prevalence of efflux mechanisms

Prevalence of efflux pumps was tested using PERMANOVA for three different data matrices: all samples, the six samples where sediment and water were collected, and the WFH gradient (Table 16). Only the WFH gradient showed a significant difference in the prevalence of efflux pumps with site being significant (p value 0.005, variation explained 8.022%). Influent had a higher amount of efflux pumps than effluent. WFH Water had lower values than both effluent and influent.

DISCUSSION

Resistance is prevalent and widespread.

This study examined the prevalence and diversity of antibiotic resistance and metal resistance genes in coastal samples—both freshwater and marine—exhibiting different environmental impacts. In examining normalized gene abundance among the five databases, sample type was significant for all database categories. The pattern of sediment having a greater percentage of resistance genes/elements for total genes recovered than water makes sense from the perspective that sediments tend to have higher numbers of bacteria and are thought to have higher levels of horizontal gene transfer activity (24). The exceptions to this pattern suggest that there are likely additional factors that are important to structuring these relationships that have not been evaluated in this study. These factors may include: taxonomic diversity within samples and what bacterial groups may more readily transfer genes, physical forcing on sediment from the water column, and sediment-water mixing/interactions at the sediment-water interface.

ARDB and CARD indicate complementary ARG in marine metagenomes.

The top 20 gene hits for both ARDB and CARD indicate complementary, but

different, antibiotic resistance groups and mechanisms of resistance with only four genes shared between the lists (BacA, dfrB6, macB, and tet34). This indicates that database choice is important and can affect the outcome of studies, and that using multiple databases to examine samples is preferable.

There are many of the same hits of top genes to other marine metagenomes, indicating that these may be players in the general marine antibiotic resistome. Several of our top 20 genes were also found in studies examining pristine (deep ocean sediments) and an anthropogenically polluted area (Pearl River Estuary) in China: macB (ARDB, CARD), acrB (ARDB), and arnA (CARD); (25) and tet34 (ARDB, CARD), tetPB (ARDB), ermF (ARDB) and mexW (ARDB); 26). One key player is bacA. bacA was found in three different marine metagenomes from around the world: in sediments in China (25), functional metagenomes of a variety of United States seawater samples (20), and mariculture systems in China (26). bacA was found more often in the polluted region, which led the authors to suggest that bacA may indicate anthropogenic activity. The mariculture system study found bacA in all studied samples. In this study, bacA was the most prevalently found resistance gene among all samples for ARDB. Within this study's results and previous literature, it seems that further research into bacA prevalence in the marine environment would be worthwhile as an indicator and as a greater understanding to its importance for marine bacteria. The bacA gene for undecaprenyl pyrophosphate phosphatase activity confers bacitracin resistance through overexpression (27).

Both ARDB and CARD illustrate that efflux pumps are a majority of resistance genes. The two databases coalesce on the hypothesis that efflux genes are prevalent in marine

samples, with ARDB's top 20 samples having 12/20 efflux genes and CARD with 11/20. The only significant variation in the amount of efflux vs. non-efflux genes present was between the WFH gradient samples. Efflux genes are prevalent in other ARG studies: 32.5% of genes were efflux pumps in a study of Chinese estuarine sediments (28) and 17.6% of those for Antarctic and marine samples (29). In a study of the relatively pristine South China Sea, efflux pumps are considered a prominent type of AR whereas a polluted area had greater diversity of ARGs/type (25). In a metagenomic study of a variety of environments, percentage of efflux gene diversity compared to the total resistance gene diversity varied dramatically from 0-74%, depending on the sample (30). In terms of specific genes, acrB and macB - both MultiDrug Resistance (MDR) efflux pumps - were found across all sites in a estuarine study of an anthropogenically impacted estuary as well as more pristine deep ocean sediments (25).

All of these results indicate an important aspect of antibiotic resistance –the mechanism of resistance. Assessing the mechanism of resistance is important to determining how easily resistances can be spread and shared. Efflux genes serve the purpose of ridding the cell of materials. In antibiotic resistance work, this is primarily focused on antibiotics, but it likely that these pumps are serving many other evolutionary, cellular needs such as detoxification, virulence, homeostasis, and signal trafficking (31). While non-specific efflux pumps were present and may be responsible for a portion of the prevalent resistance observed, known genes that are directly associated with resistance were also present. These genes were largely associated with antibiotic target alteration, and their presence suggests that some of the prevalent active resistance observed in the

cultures could have resulted either through exposure to particular antibiotics or to the acquisition of altered genes. Recent research has shown that subtherapeutic levels of antibiotics, such as those found in natural environments, can select for resistant bacteria (32, 33). In marine environments, these subtherapeutic levels could be from anthropogenic activities or could even be from the natural occurrence of antibiotics.

Little Island antibiotic resistance shows phenotypic resistance and resistance genes for similar antibiotic groups.

Little Island is a site in Falmouth, MA that has been studied in three chapters of this dissertation- Chapter 3 for active resistance in *Vibrio* bacterial cultures, Chapter 4 for active resistance in general marine heterotroph cultures, and finally, in Chapter 5 for metagenomic ARG diversity. The prevalence of phenotypic resistance does not have a direct correlation to prevalence of resistance genes (e.g. higher resistance to amoxicillin in *Vibrio* does not correspond to higher amounts of resistance genes for amoxicillin). This lack of direct correspondence is not surprising: there were temporal delays between when the culture samples and the metagenomic samples were collected, active resistance is based on specific antibiotics and the particular groups of bacteria that are able to be cultured, and metagenomics looks at the available DNA which may not represent actively expressed genes.

WFH water is distinct from wastewater effluent and influent, indicating change through groundwater process and salinity changes.

In Falmouth, the wastewater effluent is transported through groundwater to West Falmouth Harbor likely over a period of about ten years (personal communication). This area allowed for a unique test of resistance in these sites with the knowledge that samples are

not being tested all on the same time scale. Although influent/effluent were not significantly different within the databases, the addition of WFH to the comparison made site significant for all databases. In addition, the difference in number of efflux pumps between these sites was significant (SI Table 4). This illustrates that resistance and metal genes change over the course of filtration through groundwater and fits with previous work that has shown that removal of antibiotic resistance genes depends largely on the groundwater recharge system (34). Since sediment is also a diverse source of antibiotics, it is also possible that resistance genes could be picked up during the process. A study examining swine waste showed that groundwater is affected by waste, but also shows novelty from the environmental community (35).

Impact type does not structure samples, further indicating widespread resistance.
For the studied samples, there are not significant differences between impact type. This result indicates that antibiotic impacts are likely diffuse throughout the studied regions, which fits in with the results seen in Chapter 3 and Chapter 4 of this dissertation. Overall, this result may indicate that pollution (e.g. metal, antibiotic) or other anthropogenic impacts affecting the studied databases may be more widespread and less tied to particular point sources. As a recent study examining metal resistance and antibiotic resistance in mangroves eloquently described "genes involved in both heavy metals and antibiotics are ubiquitous, irrespective of the ecosystem examined" (36). It could also be that the levels seen are indicative of this area at large, representing coastal areas that have been impacted by humans.

In general, previous metagenomic studies of resistance genes have found that

similar environments group together (18, 37, 38). In a study of estuarine sediments with diverse and abundant ARG, the researchers found that anthropogenic activity contributes to resistance (28). Due to this study's results showing lack of impact, it seems that these sites lack a substantial, direct anthropogenic impact that was able to be measured here. One explanation for this lack of impact could be that legacy pollution sources, such as industrial, may not be seen in the samples examined because sampling was at the top of the water column and the sediment. Legacy pollution sources may be better represented deeper in the sediment where reduced physical forces from the overlying water can act upon it. Another explanation may be that pollution did, at one point, affect these resistance communities, but their resiliency allowed them to spring back. Previous research shows this as a possibility. In a study of extreme flooding in Colorado, flooding reduced the levels of resistance (likely by dilution), but ten months post flood, the greater abundance of resistance genes returned (39).

Marine water samples group together.

In this study, marine water samples generally grouped together while the more freshwater-influenced, river related samples were separated (Charles River, New Bedford Harbor, and Plymouth). Charles River and New Bedford Harbor sites are both in rivers so the physical flow of water may be different compared to other samples. Charles River and Plymouth had very low salinities, indicating an abundance of freshwater. These impacts could change the taxonomy and potentially even the resistances present. Provincetown, which is perhaps the most "open ocean area" and whose water is least constrained geographically, shows the highest variability on the nMDS from sample to sample.

Interestingly enough, when all water samples except for effluent and influent were tested, site was no longer significant for ARDB (genes and antibiotic) and BacMet, whereas it was for CARD and GreenGenes. This indicates that the differences between all water samples for ARDB and BacMet were being driven by effluent and influent. In an nMDS plot for the GreeGenes data, similarities can be seen between sample replicates and a range of variability for the sites is evident. These results show that regardless of the site differences indicated by taxonomy, the genes and their abundances identified by ARDB and BacMet remain similar. For BacMet, it is interesting that despite the taxonomic changes, the difference between sites is not significant. This result may lend itself towards and understanding that coastal environments may share similar metal resistances regardless of location. Another option may be that BacMet categorizes by metal and not the specific gene, so variations may occur by gene that are not being adequately captured by this examination.

Effluent and influent are distinct from other wastewater related samples.

Examining just the water samples illustrates that effluent and influent are different from each other and from the rest of the wastewater influenced samples. Influent and effluent are different from environmentally derived samples, which is not particularly surprising, as it would be expected that wastewater influent would be different from these samples. The spatial separation between influent and effluent on the nMDS plots indicate that changes do occur in the process of wastewater treatment (8, 40, 41). Effluent shows greater variability than the influent, indicating lower predictability throughout the wastewater treatment process. Effluent shows more similarity in structuring with

environmental samples that have been influenced by wastewater. With SIMPER testing, key players do change between effluent and influent for ARDB, CARD, and GreenGenes, further illustrating these changes.

In examining the wastewater influenced water only samples, there is a high degree of similarity (Figure 5B and 5C). These are all water samples that have been influenced by wastewater outfalls/groundwater flows. There are two Plymouth samples that appear to be outliers and can be seen most clearly in Figure 5C. The Plymouth outliers may be due to the fact that when sampling at low tide, the Plymouth outfall pipe was spatially removed from the rest of the Plymouth water body. The Plymouth sample may be more indicative of outflow and less so of the marine environment, which may be driving that difference.

Conclusion

This study has utilized metagenomic sequencing to show the diversity of mobile genetic elements, antibiotic and metal resistances, and taxonomic groups present in differently impacted coastal environments in Massachusetts, US. Results indicate that resistance is prevalent and widespread among a variety of coastal sites. Results also show that impact type is not significant while site and sample type are. In these coastal environments, humans and organisms that humans consume, can readily interact with water and sediment that contain resistance genes. Now that resistance genes have been found to be prevalent in these areas, the next step would be to determine the transfer rates of resistance genes from bacteria to bacteria and to humans to estimate risk potential.

References

1. **Berendonk TU**, **Manaia CM**, **Merlin C**, **Fatta-Kassinos D**, **Cytryn E**, **Walsh F**, **Bürgmann H**, **Sørum H**, **Norström M**, **Pons M-N**, **Kreuzinger N**, **Huovinen P**, **Stefani S**, **Schwartz T**, **Kisand V**, **Baquero F**, **Martinez JL**. 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 1–8.
2. **D'Costa VM**, **King CE**, **Kalan L**, **Morar M**, **Sung WWL**, **Schwarz C**, **Froese D**, **Zazula G**, **Calmels F**, **Debruyne R**, **Golding GB**, **Poinar HN**, **Wright GD**. 2011. Antibiotic resistance is ancient. Nature **477**:457–61.
3. **Bhullar K**, **Waglechner N**, **Pawlowski A**, **Koteva K**, **Banks ED**, **Johnston MD**, **Barton H a**, **Wright GD**. 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS One **7**:e34953.
4. **Baker-Austin C**, **Wright MS**, **Stepanauskas R**, **McArthur J V.** 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol. **14**:176–182.
5. **Timoney JF**, **Port J**, **Giles J**, **Spanier J**. 1978. Heavy-Metal and Antibiotic Resistance in the Bacterial Flora of Sediments of New York Bight Heavy-Metal and Antibiotic Resistance in the Bacterial Flora of Sediments of New York Bight.
6. **Mcarthur JV**, **Tuckfield RC**. 2000. Spatial Patterns in Antibiotic Resistance among Stream Bacteria : Effects of Industrial Pollution Spatial Patterns in Antibiotic Resistance among Stream Bacteria : Effects of Industrial Pollution **66**:3722–3726.
7. **Uyaguari-Díaz MI**, **Croxen MA**, **Luo Z**, **Cronin KI**, **Hartmann EM**. 2018. Human Activity Determines the Presence of Integron-Associated and Antibiotic Resistance Genes in Southwestern British Columbia.
8. **Rizzo L**, **Manaia C**, **Merlin C**, **Schwartz T**, **Dagot C**, **Ploy MC**, **Michael I**, **Fatta-Kassinos D**. 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. Sci. Total Environ. **447**:345–360.
9. **Marti E**, **Jofre J**, **Balcazar JL**. 2013. Prevalence of antibiotic resistance genes and bacterial community composition in a river influenced by a wastewater treatment plant. PLoS One **8**:1–8.
10. **Kennedy J**, **Marchesi JR**, **Dobson ADW**. 2008. Marine metagenomics : strategies for the discovery of novel enzymes with biotechnological applications from marine environments **8**:1–8.
11. **Barone R**, **Santi C De**, **Esposito FP**, **Tedesco P**, **Galati F**, **Visone M**, **Scala A Di**, **Pascale D De**. 2014. Marine metagenomics , a valuable tool for enzymes and bioactive compounds discovery **1**:1–6.
12. **Delmont TO**, **Quince C**, **Shaiber A**, **Esen ÖC**, **Lee STM**, **Rappé MS**, **Mclellan SL**, **Lücker S**, **Eren AM**. 2018. Nitrogen-fixing populations of Planctomycetes and Proteobacteria are abundant in surface ocean metagenomes. Nat. Microbiol. **3**:804–813.
13. **Coutinho FH**, **Silveira CB**, **Gregoracci GB**, **Thompson CC**, **Edwards RA**, **Brussaard CPD**, **Dutilh BE**, **Thompson FL**. 2017. Marine viruses discovered via metagenomics shed light on viral strategies throughout the oceans. Nat. Commun. **8**:1–12.
14. **Staley JT**, **Konopka a**. 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annu. Rev. Microbiol. **39**:321–

346.
15. **Rappé MS**, **Giovannoni SJ**. 2003. The uncultured microbial majority. Annu. Rev. Microbiol. **57**:369–394.
16. **Epstein SS**. 2013. The phenomenon of microbial uncultivability. Curr. Opin. Microbiol. **16**:636–642.
17. **Port JA**, **Wallace JC**, **Griffith WC**, **Faustman EM**. 2012. Metagenomic Profiling of Microbial Composition and Antibiotic Resistance Determinants in Puget Sound. PLoS One **7**.
18. **Wallace JC**, **Youngblood JE**, **Port JA**, **Cullen AC**, **Smith N**, **Workman T**, **Faustman EM**. 2018. Variability in metagenomic samples from the Puget Sound : Relationship to temporal and anthropogenic impacts 1–20.
19. **Nesme J**, **Cécillon S**, **Delmont TO**, **Monier JM**, **Vogel TM**, **Simonet P**. 2014. Large-scale metagenomic-based study of antibiotic resistance in the environment. Curr. Biol. **24**:1096–1100.
20. **Hatosy SM**, **Martiny AC**. 2015. The ocean as a global reservoir of antibiotic resistance genes. Appl. Environ. Microbiol. **81**:7593–7599.
21. **Qiagen**. 2001. Qiagen Genomic DNA Handbook.
22. **Arango-Argoty G**, **Singh G**, **Heath LS**, **Pruden A**, **Xiao W**, **Zhang L**. 2016. MetaStorm : A Public Resource for Customizable Metagenomics Annotation 1–13.
23. **Clarke KR**, **Gorley RN**. 2015. PRIMER v7 : User Manual and Tutorial. Plymouth.
24. **Heß S**, **Berendonk TU**, **Kneis D**. 2018. Antibiotic resistant bacteria and resistance genes in the bottom sediment of a small stream and the potential impact of remobilization. FEMS Microbiol. Ecol. 1–11.
25. **Chen B**, **Liang X**, **Huang X**, **Zhang T**, **Li X**. 2013. Differentiating anthropogenic impacts on ARGs in the Pearl River Estuary by using suitable gene indicators. Water Res. **47**:2811–2820.
26. **Wang J**, **Lu J**, **Zhang Y**, **Wu J**, **Luo Y**, **Liu H**. 2018. Metagenomic analysis of antibiotic resistance genes in coastal industrial mariculture systems. Bioresour. Technol. **253**:235–243.
27. **Ghachi M El**, **Bouhss A**, **Blanot D**, **Mengin-lecreulx D**, **Bacteriol CMJ**. 2004. The bacA Gene of Escherichia coli Encodes an Undecaprenyl Pyrophosphate Phosphatase Activity. J. Biol. Chem. **279**:30106–30113.
28. **Zhu Y**, **Zhao Y**, **Li B**, **Huang C**, **Zhang S**, **Yu S**, **Chen Y**, **Zhang T**, **Gillings MR**, **Su J**. 2017. Continental-scale pollution of estuaries with antibiotic resistance genes. Nat. Microbiol. **16270**.
29. **Durso LM**, **Miller DN**, **Wienhold BJ**. 2012. Distribution and Quantification of Antibiotic Resistant Genes and Bacteria across Agricultural and Non-Agricultural Metagenomes. PLoS One **7**:1–12.
30. **Fitzpatrick D**, **Walsh F**, **Walsh F**. 2016. Antibiotic resistance genes across a wide variety of metagenomes 1–8.
31. **Martinez JL**. 2003. Recent advances on antibiotic resistance genes, p. 14–32. *In* Fingerman, M, Nagabhushanam, R (eds.), Recent Advances in Marine Biotechnology.
32. **Andersson DI**, **Hughes D**. 2014. Microbiological effects of sublethal levels of

antibiotics. Nat. Rev. Microbiol. **12**:465–78.
33. **Ghosh S**, **LaPara TM**. 2007. The effects of subtherapeutic antibiotic use in farm animals on the proliferation and persistence of antibiotic resistance among soil bacteria. ISME J. **1**:191–203.
34. **B U**, **Do H**, **Ayuso-gabella MN**, **Marc MS De**, **Tandoi V**, **Levantesi C**, **Masciopinto C**, **Houtte E Van**, **Szewzyk U**, **Wintgens T**, **Grohmann E**. 2009. Quantitative PCR Monitoring of Antibiotic Resistance Genes and Bacterial Pathogens in Three European Artificial Groundwater Recharge Systems □ † **75**:154–163.
35. **Koike S**, **Krapac IG**, **Oliver HD**, **Yannarell a C**, **Chee-Sanford JC**, **Aminov RI**, **Mackie RI**. 2007. Monitoring and source tracking of tetracycline resistance genes in lagoons and groundwater adjacent to swine production facilities over a 3-year period. Appl. Environ. Microbiol. **73**:4813–23.
36. **Imchen M**, **Kumavath R**, **Barh D**, **Vaz A**, **Góes-neto A**, **Tiwari S**, **Ghosh P**, **Wattam AR**. 2018. Comparative mangrove metagenome reveals global prevalence of heavy metals and antibiotic resistome across different ecosystems 1–15.
37. **Pal C**, **Bengtsson-palme J**, **Kristiansson E**, **Larsson DGJ**. 2016. The structure and diversity of human , animal and environmental resistomes. Microbiome 1–15.
38. **Li B**, **Yang Y**, **Ma L**, **Ju F**, **Guo F**, **Tiedje JM**, **Zhang T**. 2015. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes **9**:2490–2502.
39. **Garner E**, **Wallace JS**, **Argoty GA**, **Wilkinson C**, **Fahrenfeld N**, **Heat LS**, **Zhang L**, **Arabi M**, **Aga DS**. 2016. Metagenomic profiling of historic Colorado Front Range flood impact on distribution of riverine antibiotic resistance genes 1–10.
40. **Hultman J**, **Tamminen M**, **Katariina P**, **Cairns J**, **Karkman A**, **Virta M**. 2018. Host range of antibiotic resistance genes in wastewater treatment plant influent and effluent 1–10.
41. **Yang Y**, **Li B**, **Zou S**, **Fang HHP**, **Zhang T**. 2014. Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. Water Res. **62**:97–106.

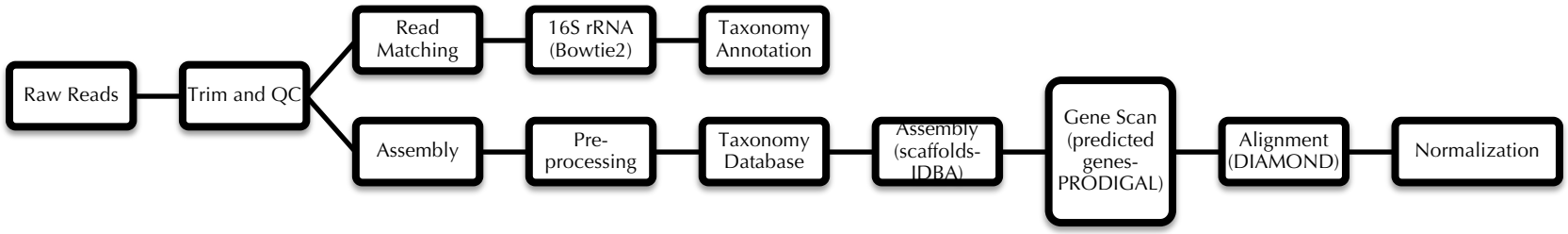


Figure 1. Figure of metagenomic analysis process.
This figure has been modified from Figure 2 of Arango-Argoty et al. 2016 (22) and shows the data processing of metagenomic data using MetaStorm.

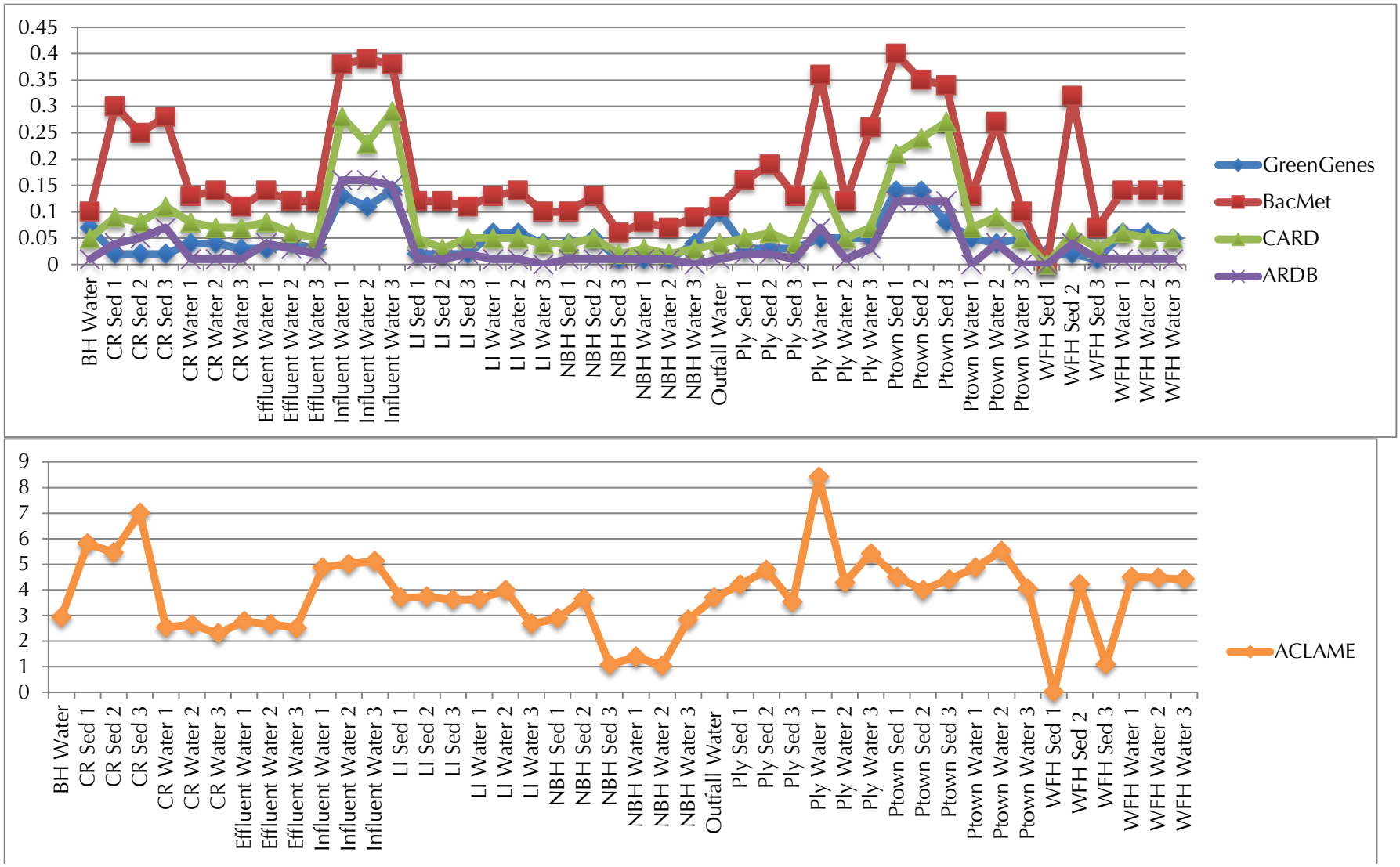


Figure 2. Percentage of total assembled genes.
This figure illustrates the percentage of total assembled genes for each database. For the top panel, GreenGenes, BacMet, CARD, and ARDB are illustrated. For the bottom panel, ACLAME is shown.

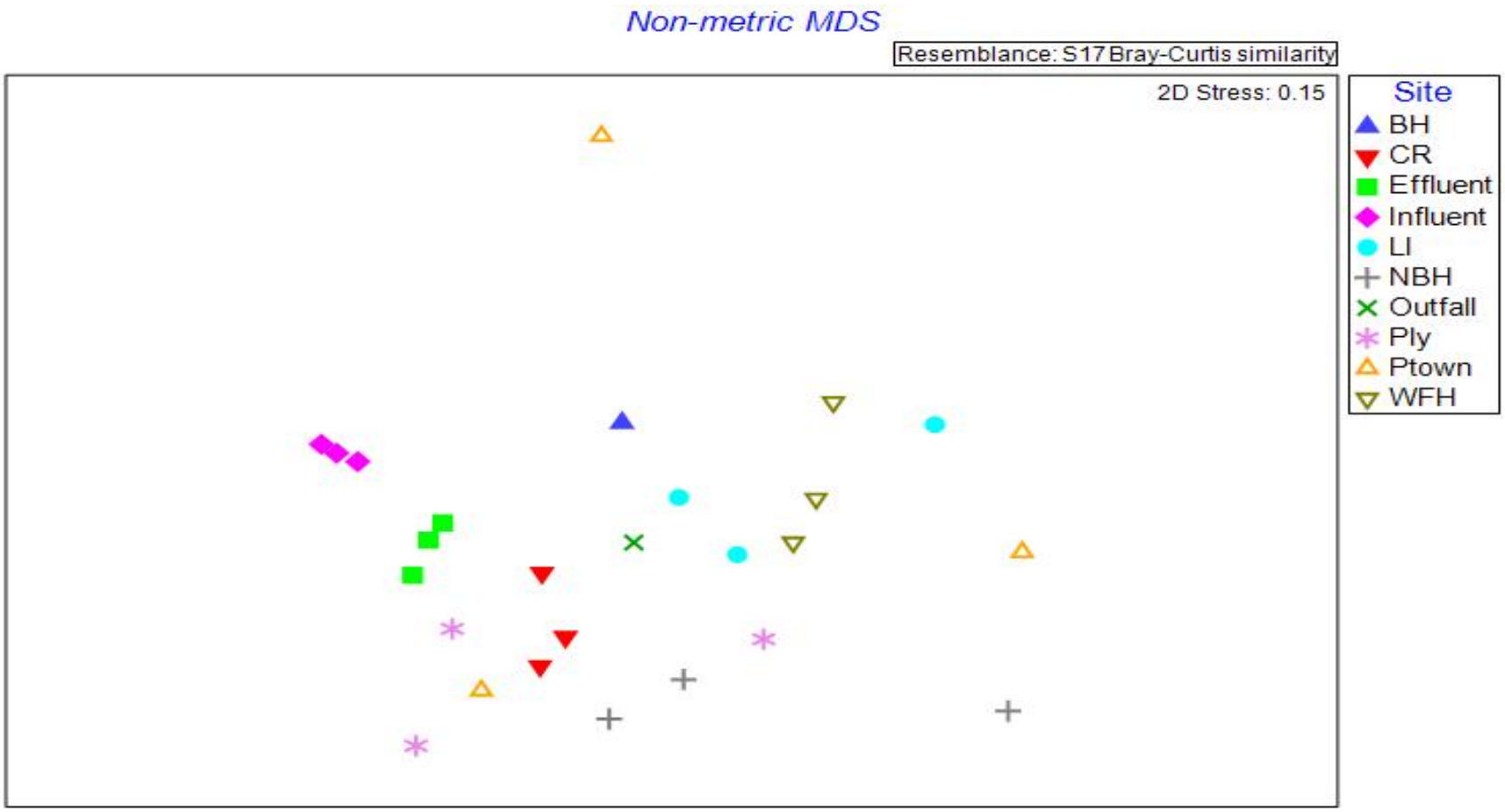


Figure 3. nMDS plot illustrating all water samples.
Data shown is for antibiotic resistance database (ARDB) and is categorized by site.

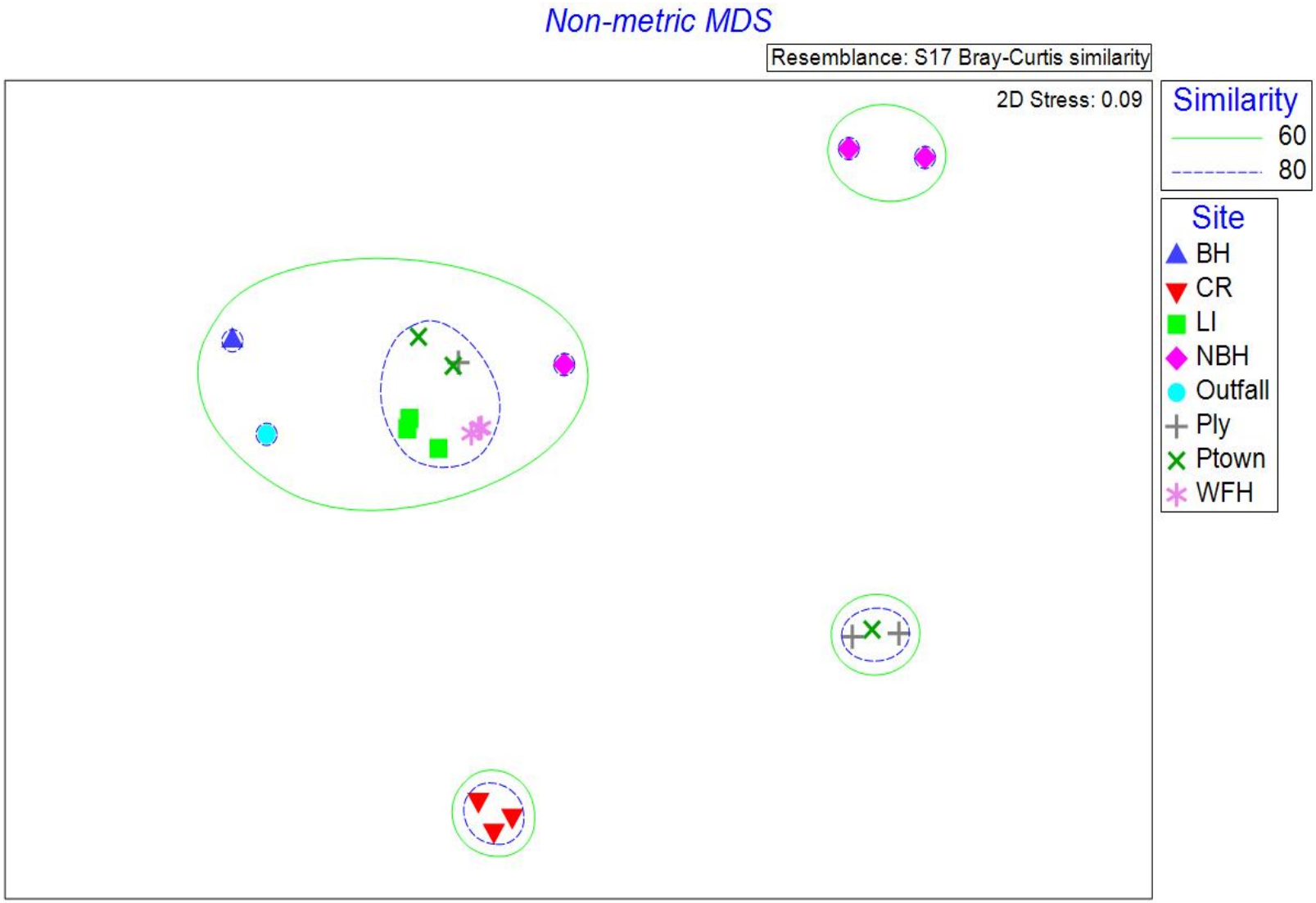
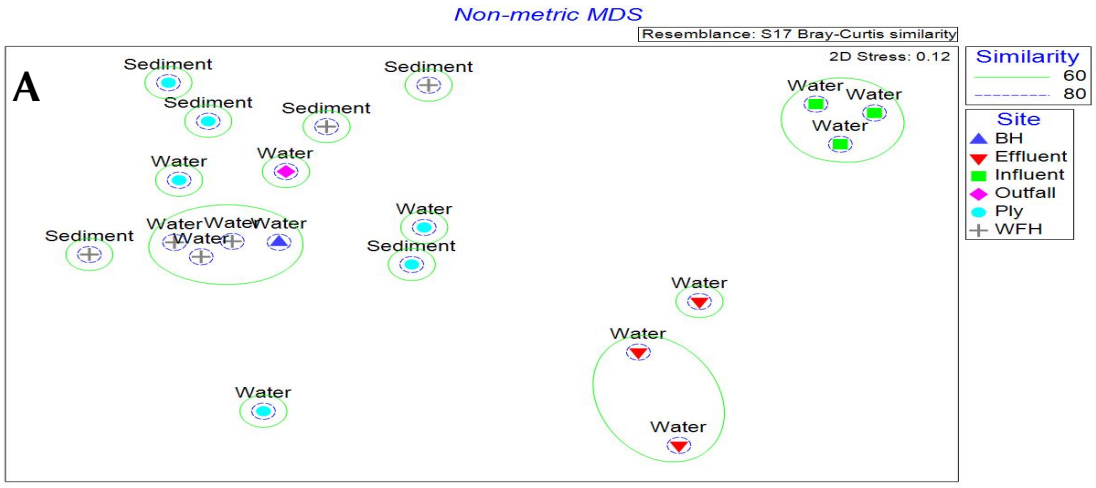
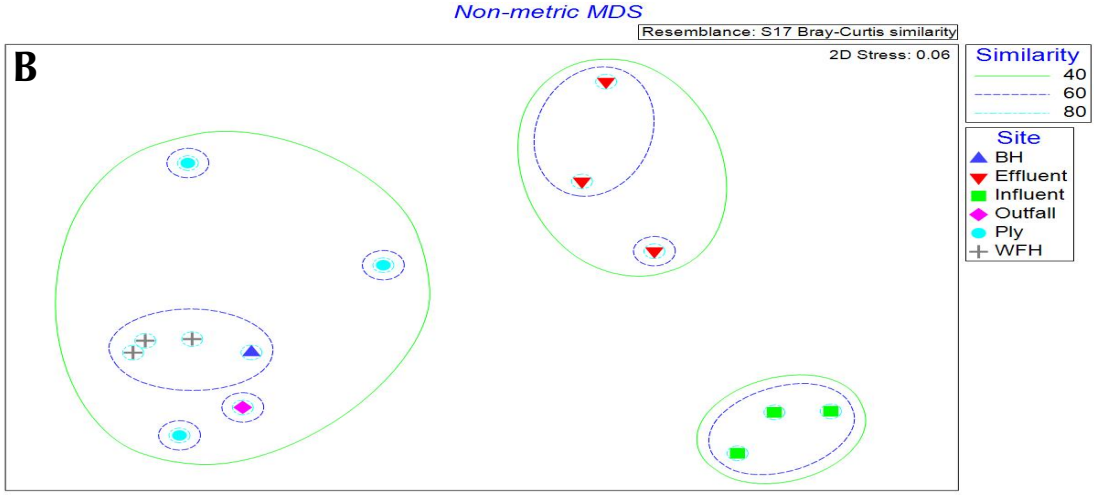
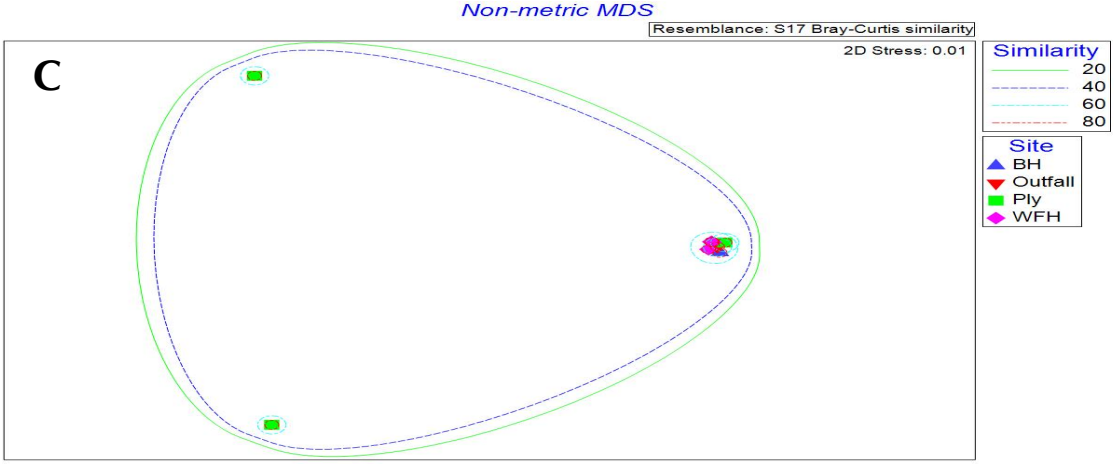


Figure 4. nMDS plot illustrating all water samples other than influent and effluent.
Data shown is for taxonomy through GreenGenes and is categorized by site.


Non-metric MDS
Resemblance: S17 Bray-Curtis similarity
A
2D Stress: 0.12
Sediment
Sediment
Sediment
Sediment
Water
Water
Water
Water
Water
Water
Water
Water
Water
Water
Sediment
Sediment
Water
Water
Water
Water
Similarity
60
80
Site
BH
Effluent
Influent
Outfall
Ply
WFH


Non-metric MDS
Resemblance: S17 Bray-Curtis similarity
B
2D Stress: 0.06
Similarity
40
60
80
Site
BH
Effluent
Influent
Outfall
Ply
WFH


Non-metric MDS
Resemblance: S17 Bray-Curtis similarity
C
2D Stress: 0.01
Similarity
20
40
60
80
Site
BH
Outfall
Ply
WFH

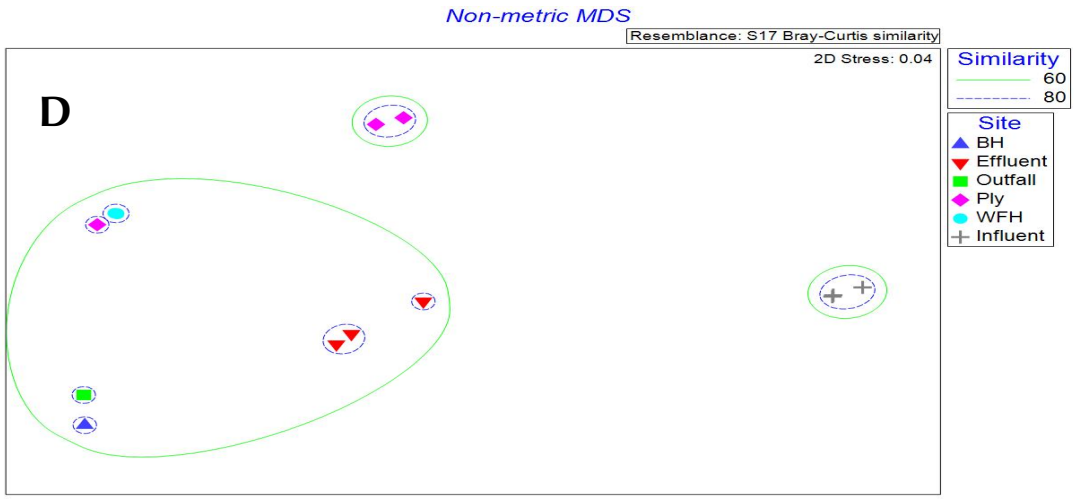


Figure 5. nMDS plot illustrating CARD gene and GreenGene wastewater samples by A.) CARD gene water and sediment samples by site (color) and by type (text), B.) CARD gene only water samples, C.) CARD gene water samples without effluent and influent., and D.) GreenGenes only water wastewater samples.

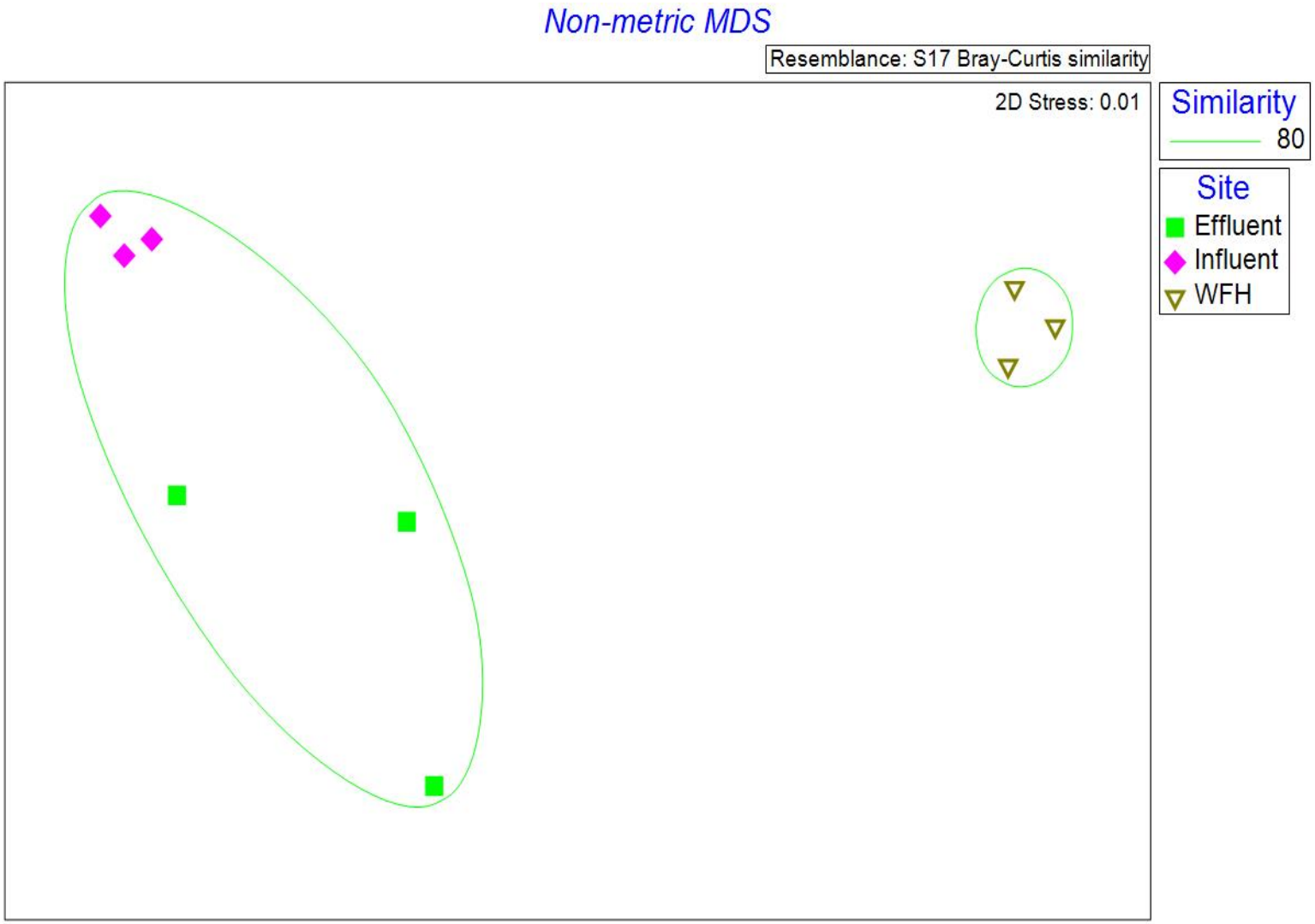


Figure 6. nMDS plot illustrating WFH gradient water samples (effluent, influent, and WFH).
Data shown is for biocide/metal resistance with BacMet data and is categorized by site.

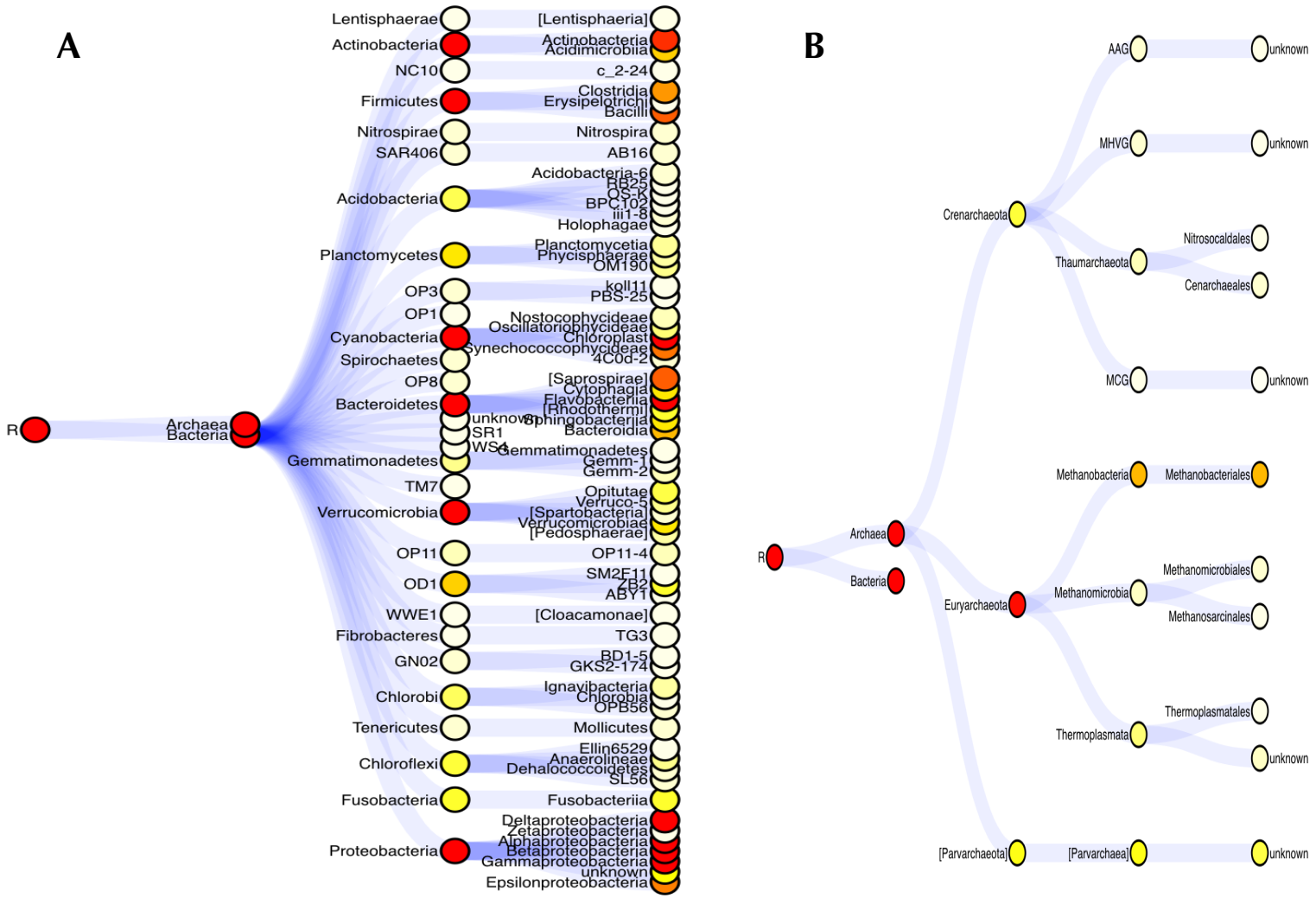


Figure 7. Prokaryotic diversity in metagenomic samples.
This figures illustrates the prokaryotic diversity illustrated from the metagenomic samples. A. Tree indicates the bacterial diversity. B. Tree indicates the archaeal diversity.

Table 1. Explanation of field sites.
Field sites used in this study indicate the abbreviation used, location, and other pertinent sampling information.

Site	Abbreviations	Location	Type	Sample collected	Sampling Date	Filters: water	Amount for metagenomes	Notes
Charles River	CR	Cambridge, MA	Industrial	Water, sediment	August 28, 2016	4- 250 mls	For each sample replicate, two filters used for total of 500 mls filtered.	freshwater
New Bedford Harbor	NBH	Acushnet, MA	Primary: Industrial Wastewater as well	Water, sediment	August 25, 2016	1- 500 mls 2- 250 ml 3- 250 ml	Sample 1- 500 mls Sample 2- 500 mls Sample 3- 500 mls	
Plymouth	Ply	Plymouth, MA	Wastewater	Water, sediment	September 2, 2016	4 -250 ml	For each sample replicate, two filters used for total of 500 mls filtered.	direct discharge of wastewater treatment to the ocean freshwater at time because water flow low and at low tide
West Falmouth Harbor	WFH	West Falmouth, MA	Wastewater	Water, sediment	September 12, 2016	4- 250 ml	For each sample replicate, two filters used for total of 500 mls filtered.	indirect wastewater treatment through groundwater and then released into WFH that takes ~10 years
Little Island	LI	Falmouth, MA	Reference	Water, sediment		4- 250 ml	For each sample replicate, two filters used for total of 500 mls filtered.	
Race Point	Ptown	Provinceto	Reference	Water,	Septembe	4- 250 ml	For each sample	

Beach		wn, MA		sediment	r 18, 2016		replicate, two filters used for total of 500 mls filtered.	
Falmouth Wastewater treatment Effluent	Effluent	Falmouth, MA	Wastewater -output	Water	October 12, 2016	3- 250 ml	Sample 1- 250 mls Sample 2- 250 mls Sample 3- 250 mls	freshwater
Influent, Falmouth Wastewater Treatment Plant	Influent	Falmouth, MA	Wastewater -input	Water	October 12, 2016	3- 100 ml samples	Sample 1- 100 mls Sample 2- 100 mls Sample 3- 100 mls	freshwater
Boston Harbor	BH		Industrial	Water	April 25, 2016	~ 3 liters	~ 3 liters	
Boston Deer Island Outfall	Outfall		Wastewater	Water	April 25, 2016	~ 3 liters	~ 3 liters	

Table 2. Available environmental characteristics from sampling sites.
Environmental characteristics collected from a YSI probe, turbidity meter, or local rain collection are shown here.

Site	Date	Time	Barometer (mmHg)	Conductivity (uS/cm)	Salinity (PSU)	Dissolved Oxygen (mg/L)	Temperature (C)	Air Temperature (C)	Previous Rainfall (in)	Turbidity (NTU)
New Bedford Harbor (NBH)	8/25/16	10:23 AM	765	45715	29.0	4.7	26.0	27.13	0	23.9
Charles River (CR)	8/28/16	11:52 AM	768	2610	1.3	9.3	27.3	26.39	0	11.6
Little Island (LI)	8/31/16	12:50 PM	761	46047	29.5	12.1	25.6	24.67	0	1.2
Plymouth (PLY)	9/2/16	7:00 AM	761	823	0.5	8.4	14.1	18.36	0.15	0.5
West Falmouth Harbor (WFH)	9/12/16	9:30 AM	768	39968	26.4	5.5	23.4	20.47	0	1.8
Race Point Beach, Provincetown (PRO)	9/18/16	9:16 AM	764	40176	29.8	8.0	18.5	23.76	0.002	1.3
Falmouth Effluent	10/12/16	9:00 AM	771	1127	0.6	4.7	20.5	NA	NA	1.6
Falmouth Influent	10/12/16	9:00 AM	771	1063	0.6	4.3	20.3	NA	NA	79.5

Table 3. Read matching summary.
Table illustrates how many reads matched for each database using the unassembled MetaStorm read matching pipeline. GreenGenes numbers include all hits to GreenGenes, regardless of taxonomic identity.

Samples	Raw Reads	Reads after QC	GreenGenes	GreenGenes % total reads after QC
BH Water	17,569,196	16,313,539	11,312	0.07
CR Sed 1	10,717,930	9,347,555	2,148	0.02
CR Sed 2	10,942,791	9,903,829	2,409	0.02
CR Sed 3	8,856,114	7,409,217	1,778	0.02
CR Water 1	12,876,089	12,095,985	4,754	0.04
CR Water 2	13,936,865	13,058,636	5,506	0.04
CR Water 3	10,284,930	9,264,552	2,836	0.03
Effluent Water 1	10,135,451	6,689,153	2,084	0.03
Effluent Water 2	10,413,694	8,808,664	3,529	0.04
Effluent Water 3	11,198,174	8,653,199	2,307	0.03
Influent Water 1	12,239,120	11,452,426	14,912	0.13
Influent Water 2	10,450,415	9,591,164	10,892	0.11
Influent Water 3	12,468,439	11,513,547	15,679	0.14
LI Sed 1	11,927,925	10,942,165	1,857	0.02
LI Sed 2	10,919,494	9,954,408	1,811	0.02
LI Sed 3	11,367,628	9,551,334	1,610	0.02
LI Water 1	6,011,514	5,794,961	3,305	0.06
LI Water 2	8,661,529	8,208,452	5,059	0.06
LI Water 3	25,019,878	23,153,017	8,272	0.04
NBH Sed 1	24,086,706	22,539,857	8,129	0.04
NBH Sed 2	12,624,416	11,693,903	5,850	0.05
NBH Sed 3	19,775,842	17,642,703	1,888	0.01
NBH Water 1	14,486,114	13,688,036	1,324	0.01
NBH Water 2	12,732,898	11,815,072	1,086	0.01

NBH Water 3	6,292,873	6,091,006	2,152	0.04
Outfall Water	8,207,017	7,865,451	7,994	0.10
Ply Sed 1	9,478,710	8,831,923	2,389	0.03
Ply Sed 2	17,325,108	15,944,565	4,514	0.03
Ply Sed 3	9,645,693	9,104,579	2,644	0.03
Ply Water 1	4,940,451	4,225,490	2,072	0.05
Ply Water 2	11,882,806	11,044,861	5,819	0.05
Ply Water 3	11,966,780	10,665,428	4,989	0.05
Ptown Sed 1	5,900,784	3,334,588	4,577	0.14
Ptown Sed 2	10,717,447	7,986,612	11,192	0.14
Ptown Sed 3	3,959,679	3,268,273	2,746	0.08
Ptown Water 1	5,238,338	4,751,025	2,375	0.05
Ptown Water 2	11,329,640	10,787,124	4,527	0.04
Ptown Water 3	10,109,892	9,567,706	4,620	0.05
WFH Sed 1	13,703,960	13,155,786	3,198	0.02
WFH Sed 2	9,873,667	9,341,867	1,951	0.02
WFH Sed 3	16,555,462	15,753,093	1,689	0.01
WFH Water 1	11,474,498	11,004,451	6,772	0.06
WFH Water 2	9,222,470	8,787,950	5,150	0.06
WFH Water 3	9,746,918	9,105,440	4,974	0.05
TARA_023_SRF_0.22-1.6	37,310,366	36,946,964	42,078	0.11

Table 4. Metagenome assembly.
Table includes metagenome results from the MetaStorm gene assembly pipeline. A Tara Oceans sample (42) is included for comparison.

Sample	Raw Reads	Input Reads after QC (after QC and assembly preprocessing)	Average read length (bps)	Assembled reads (#)	Assembled reads (%)	Scaffolds	Average scaffold length (bps)	N50 of scaffolds (bps)	Total predicted genes	Average gene length (bps)
BH Water	17,569,196	16,307,303	128	8,873,112	54.4	367,998	717	1731	444,235	494
CR Sed 1	10,717,930	9,344,234	128	290,132	3.1	29,567	630	712	41,482	387
CR Sed 2	10,942,791	9,900,291	128	339,483	3.4	36,706	610	681	50,429	382
CR Sed 3	8,856,114	7,406,448	128	245,628	3.3	22,321	648	724	32,000	397
CR Water 1	12,876,089	12,091,399	128	5,663,485	46.8	250,149	881	1,597	409,642	475
CR Water 2	13,936,865	13,053,718	128	6,249,315	47.9	273,334	890	1,604	448,550	479
CR Water 3	10,284,930	9,261,359	128	4,001,995	43.2	182,967	872	1,370	310,518	458
Effluent Water 1	10,135,451	6,686,830	128	2,607,413	39.0	98,743	823	1,204	163,807	435
Effluent Water 2	10,413,694	8,805,621	128	3,452,494	39.2	137,817	871	1,303	237,233	445
Effluent Water 3	11,198,174	8,650,027	128	3,578,187	41.4	126,728	801	1,170	207,379	428
Influent Water 1	12,239,120	11,448,274	128	4,486,556	39.2	284,720	606	1,109	312,719	443
Influent Water 2	10,450,415	9,587,841	128	3,495,244	36.5	205,405	694	1,291	264,394	445
Influent Water 3	12,468,439	11,509,288	128	4,417,443	38.4	260,986	692	1,359	330,767	451
LI Sed 1	11,927,925	10,938,312	128	363,795	3.3	56,338	558	561	72,360	357
LI Sed 2	1,0919,494	9,950,863	128	305,242	3.1	47,368	557	572	59,799	357
LI Sed 3	11,367,628	9,547,835	128	235,592	2.5	34,518	590	607	45,640	366
LI Water 1	6,011,514	5,793,830	128	1,931,129	33.3	134,313	712	928	191,012	445

LI Water 2	8,661,529	8,206,915	128	3,212,905	39.1	194,823	698	1,060	250,523	471
LI Water 3	25,019,878	23,144,390	128	9,957,572	43.0	614,719	558	1,311	620,855	444
NBH Sed 1	24,086,706	22,531,554	128	10,607,120	47.1	649,744	543	1,273	634,044	445
NBH Sed 2	12,624,416	11,689,557	128	4,358,160	37.3	265,397	645	1,445	308,821	461
NBH Sed 3	19,775,842	17,635,794	128	1,953,605	11.1	205,619	591	680	290,123	359
NBH Water 1	14,486,114	13,683,012	128	732,971	5.6	93,655	596	661	135,533	351
NBH Water 2	12,732,898	11,810,695	128	632,973	5.4	74,633	613	712	113,585	349
NBH Water 3	6,292,873	6,089,860	128	3,237,984	53.2	208,062	620	971	258,368	430
Outfall Water	8,207,017	7,863,900	128	3,944,694	50.2	168,545	815	1,101	252,318	482
Ply Sed 1	9,478,710	8,828,784	128	913,533	10.3	112,764	481	588	111,376	368
Ply Sed 2	17,325,108	15,938,428	128	2,431,131	15.3	265,766	574	670	306,696	400
Ply Sed 3	9,645,693	9,101,193	128	1,691,768	18.6	212,720	466	700	178,032	381
Ply Water 1	4,940,451	4,223,881	128	189,505	4.5	20,277	662	731	28,927	409
Ply Water 2	11,882,806	11,040,668	128	3,797,051	34.4	204,870	830	1,362	317,647	472
Ply Water 3	11,966,780	10,661,251	128	1,104,754	10.4	108,308	575	647	127,649	405
Ptown Sed 1	5,900,784	3,333,424	128	44,980	1.3	5,814	515	543	6,071	363
Ptown Sed 2	10,717,447	7,983,812	128	210,541	2.6	30,366	517	579	34,913	353
Ptown Sed 3	3,959,679	3,267,021	128	414,555	1.3	5,685	491	515	5,959	353
Ptown Water 1	5,238,338	4,749,194	128	816,078	17.2	61,485	817	1,162	99,884	446
Ptown Water 2	11,329,640	10,783,198	128	1,044,062	9.7	90,881	593	769	106,323	412
Ptown Water 3	10,109,892	9,567,706	128	2,935,215	30.7	166,086	822	1,320	261,469	460
WFH Sed 1	13,703,960	13,150,879	128	1,152,060	8.8	101,960	695	891	158,549	383
WFH Sed 2	9,873,667	9,338,611	128	2,346,231	25.1	68,786	942	1,745	118,286	476
WFH Sed 3	16,555,462	15,747,377	128	565,519	3.6	79,851	570	610	115,901	340
WFH Water 1	11,474,498	11,000,199	128	5,320,541	48.4	268,157	701	1,444	331,836	479
WFH Water 2	9,222,470	8,784,848	128	4,094,289	46.606	189,763	790	1,761	267,535	483
WFH Water 3	9,746,918	9,102,122	128	4,300,452	47.247	202,185	763	1,772	275,345	479
TARA_023	37,310,366	34,641,768	NA	16,847,647	48.6	581,069	750	898	831,320	481

SRF 0.22-1.6

Table 5. Resistance gene prevalence among samples.
The number of resistance genes are normalized to a total percentage of genes that were identified using different databases with MetaStorm.

Samples	Total genes	ACLAME genes	ACLAME % total assembled genes	BacMet genes	BacMet % total assembled genes	CARD genes	CARD % total assembled genes	ARDB genes	ARDB % total assembled genes
BH Water	444,235	13,044	2.94	437	0.10	203	0.05	28	0.01
CR Sed 1	41,483	2,406	5.80	123	0.30	36	0.09	18	0.04
CR Sed 2	50,429	2,755	5.46	126	0.25	42	0.08	27	0.05
CR Sed 3	32,000	2,240	7.00	91	0.28	36	0.11	22	0.07
CR Water 1	409,642	10,400	2.54	527	0.13	309	0.08	40	0.01
CR Water 2	448,550	11,850	2.64	610	0.14	327	0.07	57	0.01
CR Water 3	310,518	7,150	2.30	342	0.11	227	0.07	27	0.01
Effluent Water 1	163,807	4,545	2.77	222	0.14	136	0.08	69	0.04
Effluent Water 2	237,233	6,299	2.66	288	0.12	144	0.06	61	0.03
Effluent Water 3	207,379	5,207	2.51	244	0.12	106	0.05	44	0.02
Influent Water 1	312,719	15,235	4.87	1180	0.38	881	0.28	492	0.16
Influent Water 2	264,394	13,252	5.01	1030	0.39	602	0.23	432	0.16
Influent Water 3	330,767	16,945	5.12	1249	0.38	945	0.29	512	0.15
LI Sed 1	72,360	2,672	3.69	90	0.12	33	0.05	10	0.01
LI Sed 2	59,799	2,230	3.73	69	0.12	17	0.03	6	0.01
LI Sed 3	45,640	1,647	3.61	52	0.11	25	0.05	11	0.02
LI Water 1	191,012	6,936	3.63	250	0.13	92	0.05	12	0.01
LI Water 2	250,523	9,990	3.99	341	0.14	124	0.05	15	0.01
LI Water 3	620,855	16,560	2.67	596	0.10	233	0.04	22	0.00
NBH Sed 1	634,044	18,352	2.89	641	0.10	231	0.04	32	0.01
NBH Sed 2	308,821	11,272	3.65	404	0.13	152	0.05	18	0.01
NBH Sed 3	290,123	3,147	1.08	186	0.06	67	0.02	24	0.01
NBH Water 1	135,533	1,876	1.38	107	0.08	37	0.03	12	0.01
NBH Water 2	113,585	1,185	1.04	74	0.07	23	0.02	10	0.01
NBH Water 3	258,368	7,329	2.84	237	0.09	72	0.03	8	0.00
Outfall Water	252,318	9,298	3.69	273	0.11	99	0.04	19	0.01

Ply Sed 1	111,376	4,689	4.21	180	0.16	59	0.05	24	0.02
Ply Sed 2	306,696	14,636	4.77	584	0.19	176	0.06	62	0.02
Ply Sed 3	178,032	6,249	3.51	227	0.13	71	0.04	18	0.01
Ply Water 1	28,927	2,433	8.41	105	0.36	47	0.16	19	0.07
Ply Water 2	317,647	13,635	4.29	378	0.12	145	0.05	22	0.01
Ply Water 3	127,649	6,924	5.42	338	0.26	93	0.07	37	0.03
Ptown Sed 1	6,071	273	4.50	24	0.40	13	0.21	7	0.12
Ptown Sed 2	34,913	1,393	3.99	123	0.35	84	0.24	42	0.12
Ptown Sed 3	5,959	262	4.40	20	0.34	16	0.27	7	0.12
Ptown Water 1	99,884	4,869	4.87	130	0.13	65	0.07	4	0.00
Ptown Water 2	106,323	5,865	5.52	287	0.27	100	0.09	38	0.04
Ptown Water 3	261,469	10,592	4.05	272	0.10	124	0.05	9	0.00
WFH Sed 1	13,150,879	2,472	0.02	157	0.00	65	0.00	31	0.00
WFH Sed 2	118,286	4,999	4.23	379	0.32	75	0.06	44	0.04
WFH Sed 3	115,901	1,261	1.09	79	0.07	33	0.03	10	0.01
WFH Water 1	331,836	14,928	4.50	474	0.14	195	0.06	26	0.01
WFH Water 2	267,535	11,964	4.47	370	0.14	143	0.05	21	0.01
WFH Water 3	275,345	12,156	4.41	372	0.14	149	0.05	23	0.01

Table 6. Top twenty mobile genetic element hits from ACLAME and their associated descriptions.
Mobile genetic elements are important in determining transfer potential.

Name	Description
family:plasmids:1	go:0004009|ATP-binding cassette (ABC) transporter activity|OBSOLETE (was not defined before being made obsolete).
family:plasmids:3	go:0000155|two-component sensor activity|Catalysis of the phosphorylation of a specific transcription regulator in response to the presence of a particular signal substance outside the cell.;go:0000160|two-component signal transduction system (phosphorelay)|A conserved series of molecular signals found in prokaryotes and eukaryotes; involves autophosphorylation of a histidine kinase and the transfer of the phosphate group to an aspartate that then acts as a phospho-donor to response regulator proteins.;go:0000156|two-component response regulator activity|Alters the level of transcription of target genes, usually by binding to a transcription factor, when phosphorylated by a sensor that detects the presence of a particular signal substance outside the cell.
family:plasmids:6	go:0016491|oxidoreductase activity|Catalysis of an oxidation-reduction (redox) reaction, a reversible chemical reaction in which the oxidation state of an atom or atoms within a molecule is altered. One substrate acts as a hydrogen or electron donor and becomes oxidized, while the other acts as hydrogen or electron acceptor and becomes reduced.
family:plasmids:321	go:0016772|transferase activity, transferring phosphorus-containing groups|Catalysis of the transfer of a phosphorus-containing group from one compound (donor) to another (acceptor).
family:plasmids:26	go:0016491|oxidoreductase activity|Catalysis of an oxidation-reduction (redox) reaction, a reversible chemical reaction in which the oxidation state of an atom or atoms within a molecule is altered. One substrate acts as a hydrogen or electron donor and becomes oxidized, while the other acts as hydrogen or electron acceptor and becomes reduced.lua
family:plasmids:11	go:0016491|oxidoreductase activity|Catalysis of an oxidation-reduction (redox) reaction, a reversible chemical reaction in which the oxidation state of an atom or atoms within a molecule is altered. One substrate acts as a hydrogen or electron donor and becomes oxidized, while the other acts as hydrogen or electron acceptor and becomes reduced.
family:plasmids:53	go:0016491|oxidoreductase activity|Catalysis of an oxidation-reduction (redox) reaction, a reversible chemical reaction in which the oxidation state of an atom or atoms within a molecule is altered. One substrate acts as a hydrogen or electron donor and becomes oxidized, while the other acts as hydrogen or electron acceptor and becomes reduced.
family:plasmids:2	phi:0000262|toxin|Poisonous activity, especially of proteins or conjugated proteins produced by certain animals, higher plants, and pathogenic bacteria.;go:0052049|interaction with host via protein secreted by type III secretion system|An interaction with the host organism mediated by a substance secreted by the other organism by a type III secretion system. The host is defined as the larger of the organisms involved in a symbiotic interaction.
family:plasmids:9	go:0004009|ATP-binding cassette (ABC) transporter activity|OBSOLETE (was not defined before being made obsolete).
family:plasmids:9456	No description

family:plasmids:99	go:0016491|oxidoreductase activity|Catalysis of an oxidation-reduction (redox) reaction, a reversible chemical reaction in which the oxidation state of an atom or atoms within a molecule is altered. One substrate acts as a hydrogen or electron donor and becomes oxidized, while the other acts as hydrogen or electron acceptor and becomes reduced.
family:plasmids:25	go:0016152|mercury (II) reductase activity|Catalysis of the reaction: Hg + NADP+ + H+ = Hg2+ + NADPH + H+.;go:0050787|detoxification of mercury ion|Any process that reduce or remove the toxicity of mercuric ion. These include transport of mercury away from sensitive areas and to compartments or complexes whose purpose is sequestration of mercury ion and/or reduction of mercury ion (Hg[II]) to metallic mercury (Hg[0]).
family:plasmids:16	go:0004009|ATP-binding cassette (ABC) transporter activity|OBSOLETE (was not defined before being made obsolete).
family:plasmids:205	phi:0000184|transcription factor activity|Any transcription regulator activity that prevents or downregulates transcription.
family:plasmids:74	phi:0000153|DDE-based recombinase activity|Recomoinases with aDDE transposase have a related amino-acid motif (the DDE motif), which forms the active site of the transposase and is responsible for coordinating the cleavage and joining steps of transposition. Breakage of the DNA occurs at the end of the element (usually a hydrolysis) and is followed by breakage and integration at the target site (a transesterification reaction).;*aclame:function:555|IS3 family|group IS407;phi:0000136|transpositional DNA recombination|A process that moves a DNA region from one to another location in a genome via a DNA intermediate.*
family:plasmids:5648	No description
family:plasmids:64	go:0016491|oxidoreductase activity|Catalysis of an oxidation-reduction (redox) reaction, a reversible chemical reaction in which the oxidation state of an atom or atoms within a molecule is altered. One substrate acts as a hydrogen or electron donor and becomes oxidized, while the other acts as hydrogen or electron acceptor and becomes reduced.
family:plasmids:48	go:0016491|oxidoreductase activity|Catalysis of an oxidation-reduction (redox) reaction, a reversible chemical reaction in which the oxidation state of an atom or atoms within a molecule is altered. One substrate acts as a hydrogen or electron donor and becomes oxidized, while the other acts as hydrogen or electron acceptor and becomes reduced.;go:0047829|D-nopaline dehydrogenase activity|Catalysis of the reaction: N2-(D-1,3-dicarboxypropyl)-L-arginine + NADP+ + H2O = L-arginine + 2-oxoglutarate + NADPH.
family:plasmids:10905	No description
family:plasmids:665	No description

Table 7. Top twenty hits for ARDB database, an antibiotic resistance gene database.
The table includes description, antibiotic resistance group, and if the gene is considered an efflux pump.

Name	Description	Antibiotic Resistance Group	Efflux Pumps
BacA	Undecaprenyl pyrophosphate phosphatase, which consists in the sequestration of Undecaprenyl pyrophosphate.	bacitracin	Non-efflux
dfrB6	Group B drug-insensitive R67 dihydrofolate reductase, which can not be inhibited by trimethoprim.	trimethoprim	Non-efflux
KsgA	Specifically dimethylates two adjacent adenosines in the loop of a conserved hairpin near the 3'-end of 16S rRNA in the 30S particle. Its inactivation leads to kasugamycin resistance.	kasugamycin	Non-efflux
RosB	Efflux pump/potassium antiporter system. RosA: Major facilitator superfamily transporter. RosB: Potassium antiporter.	fosmidomycin	Efflux
AcrB	Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	aminoglycoside, glycylcycline, macrolide,beta_lactam,acriflavin	Efflux
CeoB	Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	chloramphenicol	Efflux
MacB	Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump. Macrolide-specific efflux system.	macrolide	Efflux
VanRA	VanA type vancomycin resistance operon genes, which can synthesize peptidoglycan with modified C-terminal D-Ala-D-Ala to D-alanine--D-lactate.	vancomycin,teicoplanin	Non-efflux
MexF	Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	chloramphenicol,fluoroquinolone	Efflux
MexW	Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	Multidrug	Efflux
MexB	Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	aminoglycoside,tigecycline, fluoroquinolone, beta_lactam, tetracycline	Efflux
AcrA	Resistance-nodulation-cell division transporter system. Multidrug resistance efflux pump.	aminoglycoside,glycylcycline,macrolide,beta_lactam, acriflavin	Efflux
MdtH	Major facilitator superfamily transporter. Multidrug resistance efflux pump.	deoxycholate, fosfomycin	Efflux
RosA	Efflux pump/potassium antiporter system. RosA: Major facilitator superfamily transporter. RosB: Potassium antiporter.	fosmidomycin	Efflux
arnA	Bifunctional enzyme that catalyzes the oxidative decarboxylation of UDP-glucuronic acid (UDP-GlcUA) to UDP-4-keto-arabinose (UDP-Ara4O) and the addition of a formyl group to UDP-4-amino-4-deoxy-L-arabinose (UDP-	polymyxin	Non-efflux

	L-Ara4N) to form UDP-L-4-formamido-arabinose (UDP-L-Ara4FN). The modified arabinose is attached to lipid A and is required for resistance to polymyxin and cationic antimicrobial peptides.		
tet34	Xanthine-guanine phosphoribosyltransferase. Mechanism detail unknown.	tetracycline	Non-efflux
BcrA	ABC transporter system, bacitracin efflux pump.	bacitracin	Efflux
tetPB	Ribosomal protection protein, which protects ribosome from the translation inhibition of tetracycline.	tetracycline	Non-efflux
EmrE	Multidrug resistance efflux pump.	aminoglycoside	Efflux
ErmF	rRNA adenine N-6-methyltransferase, which can methylate adenine at position 2058 of 23S rRNA, conferring resistance to erythromycin.	lincosamide,streptogramin_b,macrolide	Non-Efflux

Table 8. Top twenty gene matches over all samples for the CARD database.
The CARD database examines antibiotic resistance genes. The table gives a description and explanation of efflux vs. non-efflux pumps.

Name	Description	Efflux Pumps
qacH	efflux-pump-complex-or-subunit-conferring-antibiotic-resistance;fluoroquinolone-antibiotic;small-multidrug-resistance-(SMR)-antibiotic-efflux-pump;antibiotic-efflux;	Efflux
Nocardia-rifampin-resistant-beta-subunit-of-RNA-polymerase-(rpoB2)	rifampin;rifapentine;rifabutin;peptide-antibiotic;rifamycin-resistant-beta-subunit-of-RNA-polymerase-(rpoB);antibiotic-target-replacement;antibiotic-target-alteration;rifamycin-antibiotic;rifaximin;	Non-efflux
dfrB6	iclaprim;trimethoprim;brodimoprim;tetroxoprim;diaminopyrimidine-antibiotic;antibiotic-target-replacement;trimethoprim-resistant-dihydrofolate-reductase-dfr;	Non-efflux
kdpE	kanamycin-A;kdpDE;aminoglycoside-antibiotic;protein(s)-and-two-component-regulatory-system-modulating-antibiotic-efflux;antibiotic-efflux;	Efflux
msbA	nitroimidazole-antibiotic;metronidazole;ATP-binding-cassette-(ABC)-antibiotic-efflux-pump;antibiotic-efflux;efflux-pump-complex-or-subunit-conferring-antibiotic-resistance;	Efflux
mtrA	penam;antibiotic-efflux;resistance-nodulation-cell-division-(RND)-antibiotic-efflux-pump;protein(s)-and-two-component-regulatory-system-modulating-antibiotic-efflux;macrolide-antibiotic;efflux-pump-complex-or-subunit-conferring-antibiotic-resistance;penicillin;azithromycin;erythromycin;	Efflux
msrB	streptogramin-antibiotic;ATP-binding-cassette-(ABC)-antibiotic-efflux-pump;antibiotic-efflux;macrolide-antibiotic;efflux-pump-complex-or-subunit-conferring-antibiotic-resistance;	Efflux
sav1866	efflux-pump-complex-or-subunit-conferring-antibiotic-resistance;ATP-binding-cassette-(ABC)-antibiotic-efflux-pump;antibiotic-efflux;	Efflux
Streptomyces-rishiriensis-parY-mutant-conferring-resistance-to-aminocoumarin	aminocoumarin-self-resistant-parY;clorobiocin;aminocoumarin-antibiotic;novobiocin;coumermycin-A1;antibiotic-target-alteration;aminocoumarin-resistant-parY;	Non-efflux

mexK	antibiotic-efflux;triclosan;resistance-nodulation-cell-division-(RND)-antibiotic-efflux-pump;macrolide-antibiotic;efflux-pump-complex-or-subunit-conferring-antibiotic-resistance;tetracycline-antibiotic;tetracycline;erythromycin;	Efflux
pmrE	pmr-phosphoethanolamine-transferase;peptide-antibiotic;antibiotic-target-alteration;	Non-efflux
bacA	peptide-antibiotic;undecaprenyl-pyrophosphate-related-proteins;bacitracin-B;bacitracin-F;bacitracin-A;antibiotic-target-alteration;	Non-efflux
macB	efflux-pump-complex-or-subunit-conferring-antibiotic-resistance;ATP-binding-cassette-(ABC)-antibiotic-efflux-pump;antibiotic-efflux;macrolide-antibiotic;erythromycin;	Efflux
dfrA3	iclaprim;trimethoprim;brodimoprim;tetroxoprim;diaminopyrimidine-antibiotic;antibiotic-target-replacement;trimethoprim-resistant-dihydrofolate-reductase-dfr;	Non-efflux
CRP	penam;antibiotic-efflux;resistance-nodulation-cell-division-(RND)-antibiotic-efflux-pump;protein(s)-and-two-component-regulatory-system-modulating-antibiotic-efflux;norfloxacin;macrolide-antibiotic;efflux-pump-complex-or-subunit-conferring-antibiotic-resistance;oxacillin;cloxacillin;fluoroquinolone-antibiotic;erythromycin;	Efflux
novA	efflux-pump-complex-or-subunit-conferring-antibiotic-resistance;ATP-binding-cassette-(ABC)-antibiotic-efflux-pump;aminocoumarin-antibiotic;novobiocin;antibiotic-efflux;	Efflux
tet34	tetracycline-antibiotic;antibiotic-target-alteration;tetracycline-inactivation-enzyme;antibiotic-inactivation;tetracycline;	Non-efflux
vgaC	dalfopristin;pleuromutilin;ATP-binding-cassette-(ABC)-antibiotic-efflux-pump;antibiotic-efflux;pristinamycin-IIA;pleuromutilin-antibiotic;madumycin-II;griseoviridin;efflux-pump-complex-or-subunit-conferring-antibiotic-resistance;streptogramin-antibiotic;	Efflux
arnA	pmr-phosphoethanolamine-transferase;peptide-antibiotic;antibiotic-target-alteration;	Non-efflux
pmrF	pmr-phosphoethanolamine-transferase;peptide-antibiotic;antibiotic-target-alteration;	Non-efflux

Table 9. Top 20 metal resistance groups from BacMet, a metal resistance databases.

BacMet
Others
Mercury
Copper
Arsenic
Chromium
Tellurium
Selenium
Zinc
Cobalt
Nickel
Iron
Silver
Lead
Antimony
Cadmium
Manganese
Magnesium
Tungsten
Molybdenum
Gold

Table 10. Top 20 prokaryotic genera from GreenGenes, a taxonomy database.

Genus	Domain
unknown	unknown
Sodalis	Bacteria
Arcobacter	Bacteria
unknown	unknown
unknown	unknown
Flavobacterium	Bacteria
CandidatusPortiera	Bacteria
Bacteroides	Bacteria
Acinetobacter	Bacteria
OM60	Bacteria
Octadecabacter	Bacteria
Coraliomargarita	Bacteria
ACK-M1	Bacteria
Sulfurospirillum	Bacteria
Tolumonas	Bacteria
HTCC2207	Bacteria
Hydrogenophaga	Bacteria
Synechococcus	Bacteria
Polaribacter	Bacteria
Fluviicola	Bacteria

Table 11. PERMANOVA results examining gene abundance.
Nested PERMANOVA tests examining impact type, site, and sample type were used to test significance. Abundance values were normalized to the total number of genes.

	ACLAME	ARDB	BacMet	CARD	GreenGenes
Test Results	Impact: 0.516 (-3.8193) Site: 0.447 (4.2197) **Type: 0.005 (14.746)** Residual: 20.341 Pooled Impact **Type: 0.006 (14.746)** Residual: 20.341	Impact: 0.69 (-14.04) Site: 0.113 (25.772) **Type: 0.001 (24.314)** Residual: 18.038 Pooled Impact **Type: 0.001 (24.314)** Residual: 18.038	Impact: 0.739 (-9.6711) Site: 0.183 (14.412) **Type: 0.005 (15.949)** Residual: 20.627 Pooled Impact **Type: 0.006 (15.949)** Residual: 20.627	Impact: 0.892 (-13.914) Site: 0.066 (23.067) **Type: 0.004 916.739)** Residual: 20.358 Pooled Impact **Type: 0.004 (16.739)** Residual: 20.358	Impact: 0.331 (7.911) Site: 0.485 (2.5611) **Type: 0.001 (24.989)** Residual: 15.936

Table 12. Results of PERMANOVA tests for a variety of data subsets.
ARDB-genes and CARD-genes indicate the data matrix with each entry being an individual gene. ARDB-antibiotics and CARD-antibiotics have been condensed into antibiotic groups. Bolded values indicate significance.

Database	**All Sample Matrix**	**All Water Samples Matrix (BH, CR, Effluent, Influent, LI, NBH, Outfall, Ply, Ptown, WFH)**	**All Water Samples No Effluent/Influent (BH, CR, LI, NBH, Outfall, Ply, Ptown, WFH)**	**6 nested setup (CR, LI, NBH, Ply, Ptown, WFH)**	**Wastewater Water + Sediment (BH, Effluent, Influent, Outfall, Ply sed + water, WFH sed + water)**	**Wastewater Water (BH, Effluent, Influent, Outfall, Ply water, WFH water)**	**Influent vs. Effluent (Influent, Effluent)**	**WFH Gradient (Effluent, Influent, WFH Water)**
	PERMANOVA type nested in site nested in impact type	PERMANOVA site nested in impact type	PERMANOVA site nested in impact type	PERMANOVA type nested in site nested in impact type	PERMANOVA type nested in site nested in impact type	PERMANOVA site nested in impact type	PERMANOVA by site	PERMANOVA by site
ARDB genes	Impact: 0.196 (10.21) Site: 0.06 (20.018) **Type: 0.001 (25.406)** Residual: 43.925	Impact; 0.192 (12.142) **Site: 0.001 (30.572)** Residual: 44.602	Impact: 0.214 (12.02) Site: 0.105 (17.03) Residuals: 50.023	Impact: 0.411 (-2.8875) Site: 0.146 (14.07) **Type: 0.001 (23.734)** Residuals: 46.646 Pooled impact **Type: 0.002 (23.734)** Residuals: 46.646	Impact: 0.223 (15.862) Site: 0.202 (18.419) **Type: 0.001 (32.448)** Residual: 36.38	Impact: 0.468 (10.606) **Site: 0.001 (38.585)** Residual: 37.418	Site: 0.094 (41.788) Residual: 21.229	**Site: 0.006 (47.04)** Residuals: 30.103
ARDB - antibiotic	Impact: 0.162 (11.43) Site: 0.221(13.136) **Type: 0.001 (25.44)** Residual:	Impact: 0.093 (14.393) **Site: 0.002 (23.587)** Residual: 35.382	Impact: 0.279 (10.472) Site: 0.108 (16.149) Residual: 39.868	Impact: 0.34 (7.6838) Site: 0.26 (9.4476) **Type: 0.001 (24.338)** Residuals: 37.3	Impact: 0.496 (-8.3442) Site: 0.398 (9.229) **Type: 0.001 (31.233)** Residual:	Impact: 0.718 (-12.487) Site: 0.001 (30.004) Residual: 29.618 Pooled	Site: 0.101 (30.629) Residual: 15.461	**Site: 0.005 (35.492)** Residuals: 20.94

	35.024				29.021 Pooled impact **Type: 0.001** **(31.233)** Residual: 29.021	impact Pooled 0.001 (29.204) Residual: 29.618		
BacMet	**Impact: 0.049** **(9.5955)** Site: 0.895 (-11.181) **Type: 0.001** **(18.029)** Residual: 16.205 Pool site Impact: 0.11 (7.9805) Residual: 16.205	Impact: 0.105 (9.9685) **Site: 0.007** **(11.718)** Residual: 14.081	Impact: 0.093 (9.2251) Site: 0.325 (4.4263) Residual: 15.758	Impact: 0.541 (1.9182) Site: 0.774 (-8.739) **Type: 0.001** **(17.695)** Residuals: 17.272 Pooled Impact: **Type: 0.001** **(17.695)** Residuals: 17.272	**Impact: 0.023** **(19.356)** Site: 0.973 (-16.78) **Type: 0.001** **(21.503)** Residuals: 12.893 Pool site **Impact: 0.037** **(18.809)** Residual: 12.893	Impact: 0.138 (13.767) **Site: 0.004** **(15.237)** Residual: 10.494	Site: 0.099 (8.1492) Residual: 6.9431	**Site:: 0.003** **(20.041)** Residuals: 6.3683
CARD - antibiotic	Impact: 0.295 (7.4159) Site: 0.078 (18.841) **Type: 0.001** **(23.284)** Residual: 36.466	Impact: 0.407 (1.3705) **Site: 0.001** **(31.16)** Residual: 30.146	Impact: 0.666 (-6.0564) **Site: 0.017** **(16.603)** Residual: 32.485 Pooled impact Pooled: 0.015 (15.651) Residuals: 32.485	Impact: 0.679 (-5.7476) Site: 0.304 (7.1015) **Type: 0.001** **(22.234)** Residuals: 38.382 Pooled Impact **Type: 0.001** **(22.234)** Residuals: 38.382	Impact: 0.568 (-9.1313) **Site: 0.026** **(27.141)** **Type: 0.013** **(20.412)** Residual: 32.106 Pooled impact **Type: 0.011** **(20.412)** Residual: 32.106	Impact: 0.621 (-12.02) **Site: 0.001** **(37.061)** Residual: 28.149 Pool impact Pooled: 0.001 (36.464) Residual: 28.149	Site: 0.101 (38.307) Residual: 21.664	**Site: 0.007** **(45.932)** Residuals: 19.434
CARD - gene	Impact: 0.367 (5.8597) Site: 0.078 (19.34)	Impact: 0.373 (2.0516) **Site: 0.001** **(33.587)**	Impact: 0.675 (-8.8519) **Site: 0.01** **(19.513)**	Impact: 0.647 (1.392) Site: 0.595 (-5.7921)	Impact: 0.534 (-9.8907) Site: 0.07 (28.342)	Impact: 0.938 (-27.018) **Site: 0.001** **(45.091)**	Site: 0.094 (44.227) Residual: 26.175	**Site: 0.006** **(49.961)** Residuals: 23.282

	Type: 0.001 (25.505) Residuals: 39.138	Residual: 32.975	Residual: 34.948 Pool Impact **Site: 0.005 (17.868)** Residual: 34.948	**Type: 0.001 (24.566)** Residuals: 40.901 Pooled site Impact: 0.48 (-3.0406) Residuals: 40.901	**Type: 0.005 (24.904)** Residual: 34.763 Pooled impact **Type: 0.002 (24.904)** Residuals: 34.763	Residual: 31.502 Pooled impact **Pooled: 0.001 (39.522)** Residuals: 31.502		
GreenGenes	Impact: 0.178 (10.784) Site: 0.136 (16.819) **Type: 0.001 (28.752)** Residual: 22.031	Impact: 0.338 (7.2045) **Site: 0.001 (30.155)** Residual: 22.551	Impact: 0.189 (10.475) **Site: 0.001 (22.291)** Residual: 24.387	Impact: 0.254 (7.6726) Site: 0.105(12.453) **Type: 0.001 (28.524)** Residuals: 22.904	Impact: 0.186 (18.223) Site: 0.165 (19.983) **Type: 0.001 (26.497)** Residual: 21.966	Impact: 0.206 (14.293) **Site: 0.039 (22.099)** Residual: 31.282	Site: 0.102 (41.997) Residual: 15.811	**Site: 0.004** (40.487) Residual: 13.241

Table 13. Testing at Little Island.

Little Island testing across Chapter 3 (*Vibrio*), Chapter 4 (heterotrophs), and Chapter 5 (coastal metagenomes) for comparison. *Vibrio* testing of amoxicillin also included clavulanic acid, a beta lactam. The coastal metagenome categories come from ARDB.

		Observed % Resistance			
Antibiotic	**Antibiotic Type**	***Vibrio***	**Heterotroph**	**Coastal Metagenomes**	**% Resistance Genes**
Amoxicillin	Penicillin	29.4	36	Penicillin	0.3
Ciprofloxacin	Quinolones	30	42		
Doxycycline	Tetracycline	4.2	90.7	Tetracycline	13.5
Erythromycin	Macrolide		29.6	Macrolide	16.9
Oxytetracycline	Tetracycline	5.8			
Trimethoprim	Trimethoprim	47.5		Trimethoprim	1.6
				Chloramphenicol, Fluoroquinolone	0.3
				Fosmidomycin	1.1
				Aminoglycoside,Glycylcycline,Macrolide,Beta_Lactam,Acriflavin	1.2
				T_Chloride,Acriflavine,Puromycin	2.1
				Chloramphenicol	4.3
				Streptogramin_A	4.7
				Multidrug	7.6
				Kasugamycin	15.2
				Lincosamide,Streptogramin_B,Macrolide	15.4
				Bacitracin	15.7

Table 14. SIMPER results for sample type.
SIMPER tests were completed through PRIMER to test each database for the most important components of the top 10 genes/ groupings for each database.

	ACLAME	ARDB	BacMet	CARD	GreenGenes
Water	Avg similarity: 86.71 Plasmids 1, 321, 205, 6, 11, 9456	Avg similarity: 40.97 BacA, MacB	Avg similarity: 78.79 Others, Copper, Chromium, Tellurium, Arsenic	Avg similarity: 58.17 rpoB2, pmrE, msrB	Avg similarity: 68.42 Unknown, *Sodalis*
Sediment	Avg similarity: 80.53 Plasmids 1, 321, 205, 11, 6	Avg similarity: 41.51 BacA, MacB	Avg similarity: 71.90 Others, Copper, Arsenic, Chromium, Tellurium	Avg similarity: 51.79 rpoB2, msbA	Avg similarity: 63.09 Unknown, *Sodalis*
Average dissimilarity	17.16	58.18	26.04	47.29	36.06

Table 15. SIMPER results for site.
SIMPER tests were completed through PRIMER to test each database for the most important components of the top 10 genes/ groupings for each database.

Sample	ACLAME	ARDB	CARD	BacMet	GreenGenes
BH Water	Too few samples	Too few samples	Too few samples	Too few samples	Too few samples
CR Total	82.66 Plasmids 1, 321, 205, 6, 11	68.09 BacA	58.15 rpoB2	87.32 Others, Arsenic, Copper, Chromium	83.93 Unknown1, Unknown2
CR Water	96.27 Plasmids 1, 321, 9456, 5648, 6, 205	71.44 BacA	89.95 rpoB2, mtrA, Streptomyces	94.85 Others, Copper, Arsenic, Chromium, Iron	95.09 Unknown1, *Flavobacterium*
CR Sediment	87.70 Plasmids 1, 9, 205, 6, 321	64.66 BacA	50.60 rpoB2	88.48 Arsenic, others, Chromium, Copper	95.10 Unknown1, Unknown2
Effluent Total	93.01 Plasmids 1, 6, 321, 26, 11, 53	88.12 BacA, dfrB6	71.88 rpoB2, qacH, msbA	88.90 Others, Arsenic, Copper, Mercury, Chromium, Tellurium	78.67 Unknown1, *Sodalis*
Influent Total	96.49 Plasmids 1, 205, 321, 5648, 9	89.36 BacA, AcrB, RosA, KsgA	87.95 pmrE, CRP, qacH, rpoB2	95.97 Others, Arsenic, Copper, Zinc, Mercury, Tellurium	93.14 *Arcobacter*, unknown1
LI Total	91.96 Plasmids: 1, 6, 205, 9, 321, 11	46.71 MacB, BacA	57.55 rpoB2, msrB	74.67 Others, Chromium, Tellurium, Selenium, Copper	87.88 Unknown1, *Sodalis*
LI Water	95.19 Plasmids 1, 6, 11, 205, 321, 9	50.20 KsgA, MacB, BacA	78.05 rpoB2, msrB, pmrE	93.96 Others, Chromium, Tellurium, Selenium, Copper	90.70 Unknown1, *Sodalis*
LI Sediment	90.98 Plasmids 1, 205, 9, 6, 321, 11	46.80 MacB, BacA	45.31 rpoB2, sav1866	88.74 Others, Arsenic, Chromium, Tellurium, Selenium	92.77 Unknown1, *Sodalis*
NBH Total	80.42 Plasmids 1, 205, 321,	40.71 BacA, MexW, MacB	53.49 rpoB2,	74.67 Copper, Others,	76.05 Unknown1, Unknown3

	11, 9456		Streptomyces	Chromium, Arsenic, Tellurium	
NBH Water	77.55 Plasmids 1, 205, 321, 11	25.19 BacA, arnA, MexW	31.70 rpoB2	69.02 Copper, Chromium, Arsenic, others, Tellurium	76.91 Unknown1, Unknown3
NBH Sediment	82.64 Plasmids 1, 321, 205, 11, 9456	49.11 BacA, MacB	70.40 rpoB2, Steptomyces	73.94 Others, Copper, Chromium, Tellurium, Selenium	75.97 Unknown1
Outfall Total	Too few samples	Too few samples	Too few samples	Too few samples	Too few samples
Ply Total	90.94 Plasmids 1, 6, 9, 321, 11, 205	72.64 BacA	60.81 rpoB2, pmrE, msbA	79.71 Others, Copper, Arsenic, Chromium, and Tellurium	87.07 Unknown1, Unknown2
Ply Water	90.65 Plasmids 1, 6, 321, 11, 9, 205	61.18 BacA	52.01 rpoB2, msrB, pmrE	81.13 Others, Arsenic, Tellurium, Chromium, Copper	85.36 Unknown1, Unknown3
Ply Sediment	94.64 Plasmids 1, 6, 9, 11, 321	80.03 BacA	70.95 rpoB2, msbA, pmrE	87.27 Copper, Others, Arsenic, Zinc, Chromium	94.85 Unknown1, Unknown2
Ptown Total	78.99 Plasmids 1, 321, 11, 6, 26, 205	19.75 Tet34, AcrB	47.63 rpoB2, msrB	61.65 Others, Copper, Arsenic, Tellurium	53.03 *Sodalis*, Unknown1
Ptown Water	92.87 Plasmids 1, 6, 321, 11, 5648, 205	14.21 MacB	74.67 rpoB2, msrB, pmrE	81.19 Others, Tellurium, Chromium, Arsenic	76.13 Unknown1, *Sodalis*
Ptown Sediment	68.39 Plasmids 1, 321, 11, 26	36.94 AcrB	26.08 rpoB2	63.17 Copper, Others, Cobalt	95.35 *Sodalis*
WFH Total	85.12 Plasmids 1, 205, 321,9456, 6, 5648	41.20 BacA, MexW	68.91 rpoB2, pmrE, msrB	69.95 Others, Copper, Arsenic, Chromium, Tellurium	79.98 Unknown1, unknown2

WFH Water	96.62 Plasmids 1, 6, 321, 205, 11, 9	62.69 MexW, MacB	88.52 rpoB2, pmrE, msrB	94.73 Others, Chromium, Tellurium, Selenium, Copper	95.66 Unknown1, *Sodalis*
WFH Sediment	84.56 Plasmids 1, 205, 321, 9456, 5648	54.46 BacA	58.89 rpoB2, dfrA3, msbA	72.72 Copper, Arsenic, Others, Zinc, Chromium	85.89 Unknown1, Unknown3

PERMANOVA tests were run on the percentage of CARD genes with "efflux" in their name.
This table illustrates tests to determine if there is significance of percentage of efflux genes between samples.

Name	All	Six Main Samples	WFH Gradient
Samples included	All Samples	CR, LI, NBH, Ply, Ptown, WFH Sediment + water	Effluent, Influent, WFH Water
Test type	PERMANOVA type nested in site nested in impact type	PERMANOVA type nested in site nested in impact type	PERMANOVA by site
Results	Impact: 0.286 (2.8216) Site: 0.535 (-1.4378) Type: 0.205 (3.9668) Residual: 9.5236 Pool site Impact: 0.228 (2.7371) Residual: 9.5236	Impact: 0.396 (1.9965) Site: 0.32 (3.1517) Type: 0.374 (2.2871) Residual: 10.459	**Site: 0.005 (8.022)** Residual: 2.6004

CHAPTER 6 Conclusion

Contributions from this Dissertation to the Environmental Antibiotic Resistance Field

Antibiotic resistance is widespread and persistent in coastal marine environment.

This dissertation has illustrated that antibiotic resistance is widespread and persistent in coastal marine environments, contributing to the concept that antibiotic resistance is a natural occurrence. For the sites studied on Cape Cod, there were not resistance differences based on perceived human or environmental impacts (for cultured bacteria seen in Chapter 3 or 4; for coastal metagenomic samples seen in Chapter 5). This result was surprising because other studies have illustrated impact differences (1–3). The lack of an effect in this work suggested that the impacts were not substantial, the environment recovered rapidly, or a combination of both. Further, the lack of human impact effect may illustrate that the coastal ocean has already reached saturation to antibiotics before the study period. Even in the absence of significant impact, antibiotic resistance was found at all of the local study sites and persisted throughout the seasons with little change in the overall percentage recovered. It may be possible that prevalence of antibiotic resistance is fairly homogenous throughout regions of the world, other than through comparisons of highly polluted point sources (a lake with effluent from antibiotic production in India or polluted beaches with fecal contamination in Brazil).

This work has also shown that resistance can be prevalent, even in what could be defined as relatively low impact areas; more isolates were resistant to at least one antibiotic than sensitive to all antibiotics. Additionally, specific resistance was not associated with a single strain/OTU, but rather individuals in an OTU hosted a collection of antibiotic resistance types. This suggests some flexibility or exchange of antibiotic

resistance between bacteria and supports the concept of the natural environment as a reservoir of resistance (4–10). As a reservoir, the marine environment can act as a region for exchange of genes between both pathogenic and non-pathogenic activity and in this way, potentially be a source of future clinically important resistances (11, 12). This dissertation provides increased evidence for One Health, that the environment, animals, and humans are all interconnected and their health are all intertwined (13, 14).

The metagenomic analysis of water and sediment from six sites revealed the presence of a diverse collection of genes and elements associated with resistance to antibiotics. This substantially expands a growing body of knowledge regarding the types of resistance genes present in natural environments, particularly those with low anthropogenic impacts. It also supported the observed prevalence of active resistance by the presence of general resistance genes (e.g. efflux pumps) as well as specific genes (e.g. target modification). Perhaps most importantly, the presence of plasmids and insertion elements in the metagenomes implies the potential for movement of some resistance genes between bacteria. This makes the transfer of these genes into human associated bacteria a real possibility.

The prevalence of resistance in the marine environment provides support for the concept that there are diverse uses of antibiotics besides killing other bacteria. Instead, it is likely that antibiotics and resistance provide other important ecological and evolutionary uses such as cellular signaling or attenuating cell interactions (15). Examining the microbial perspective and scales would provide a greater understanding of

this, instead of assuming that antibiotics are used for destruction, which is an anthropogenic-centric perspective.

Future directions

Test marine environments on a distinct scale (e.g. mesocosms) to see results of antibiotic resistance and changes.
Natural environments are affected by a collection of different impacts that can be difficult to discriminate. Therefore, it may be useful to create mesocosms to study distinct anthropogenic impacts on the marine environment. Direct studies such as this could uncover how different types of pollution change the environmental resistance profiles and what practices cause significant increases in resistances relevant to human disease treatment.

Determine evolutionary roles of efflux pumps in the marine environment.
Efflux pumps are an interesting type of antibiotic resistance, and just like antibiotics, likely have many other cellular purposes: resistance to toxic metals/solvents, colonization processes, homeostasis and detoxification, virulence, and cell to cell signaling (16). Understanding efflux pumps larger purpose in all environments, and particularly in the marine environment is an open question. Greater understanding of the evolutionary process of efflux genes could be explored. This study would be interesting from a basic science standpoint to understand more about the structure of marine bacterial cells and the evolution of efflux genes. It could also be applied to produce mechanisms to work around efflux pumps clinically and provide a better understanding of their future evolution and how that may affect clinical resistance.

Determine actual risks, if any, of human exposure to environmental antibiotic resistance genes and antibiotic resistant bacteria.

In terms of human exposure, the pressing future direction is if prevalence of resistance provides adverse outcomes to humans and how to quantify that risk. There are two main knowledge gaps that would need to be addressed to determine what risk, if any, is present. One knowledge gap is gene transfer between environmental bacteria and those associated with humans (commensals and pathogens). Knowledge of transfer abilities and rates between one marine bacterium to another, a non-pathogenic bacteria to pathogen, and from pathogenic/non-pathogenic bacteria to human-associated microbiota, such as those present in skin or in the gastrointestinal tract, are necessary to begin estimating the actual risk of obtaining a resistance gene from the environment. The other knowledge gap is if transfer rates are proved to be possible, examination of adverse health impacts from the transfer into the human body would need to be determined. This would likely be hard to study as it would be necessary to show that ingestion of resistant bacteria or resistance genes later prove harmful, and this harm would need to be a certain level to necessitate action. The consumption of aquatic organisms is a viable route of exposure to antibiotic resistance for humans. In this dissertation it was shown that eating raw shellfish could result in exposure to bacteria that actively express antibiotic resistance. Even further, water associated wildlife had higher AR compared to terrestrial animals in the African savanna (17). For humans, this may mean that water-derived food might have higher levels of resistance to consider.

Standardization within the antibiotic resistance community is necessary to allow monitoring of resistance.

In terms of addressing the growing antibiotic resistance crisis, it is necessary to create standardized methods for the environmental antibiotic resistance community to allow effective comparison and monitoring to take place (8, 18). Much like clinical microbiologists can compare resistances across regions and hospitals, ideally so should environmental microbiologists across biomes and geographic boundaries. The methods should ideally be inexpensive and require little specialized machinery, so they can be utilized by all countries, regardless of their resources. Bacteria do not know geographic boundaries and if the goal is the continued use of antibiotics, there is a need to work together for the common good.

To work together across geographic boundaries, a mechanism to share and compare these results is necessary. To allow for comparisons between studies, at a minimum, published papers should make their raw data accessible. In addition, standards of how to report resistance (e.g. whether total resistance includes intermediate resistance) would be helpful. A recent paper created a dashboard application to share resistance data that seems promising to compare data across clinical and natural environments (19).

In past years, focus on particular organisms and types of antibiotics/resistance for monitoring have been discussed (20–23). These conversations should continue, but not at the expense of beginning to create a program. The O'Neill report estimates that 10 million lives per year and a cumulative 100 trillion USD will be affected by AR in 2050 (24). Even though the exact number of deaths is being debated (25), the discrepancy is not with the reality that there will be an effect of antibiotic resistance; instead, the discrepancy is to

what extent will humans be impacted. Therefore, the time for forward thinking action is now.

Action is necessary to protect the use of antibiotics.

This dissertation adds to the large amount of evidence indicating that action is necessary to protect antibiotics (24, 26–29). Clinical microbiology has already shown this, in great detail, and lately, environmental microbiology has been further illustrating that resistance is prominent in what were before believed to be relatively pristine regions. Together, these allow for a robust amount of knowledge that changes must be made if humans want to continue to use the wonders of antibiotics.

Overall, areas of focus to protect AR would be to reduce antibiotic use, increase antibiotics in the pipeline, and decrease resistance (29). Reducing antibiotic use would not only affect humans, but their environments and food consumption. A recent paper showed that pharmaceutical concentrations in a stream carry up the food chain and allow organisms such as trout and platypus to ingest dosages of a given pharmaceutical that would be considered a percentage of the daily human dose (30). An environmental study in Botswana showed that humans impact flow of antibiotic resistance and simply reducing antibiotic use in agriculture likely will not have large benefits due to transmission and flow of resistance (31). Collignon et al. 2018 illustrates that simply a reduction of antibiotics cannot just be done to address AR as contagion and other public health infrastructure is important (32). Changes would likely necessitate policy change on the national and global stages. Policy makers would be well served to include impacts of climate change in these policies as its impacts will change resistance outcomes (33).

References

1. **Bengtsson-Palme J**, **Boulund F**, **Fick J**, **Kristiansson E**, **Joakim Larsson DG**. 2014. Shotgun metagenomics reveals a wide array of antibiotic resistance genes and mobile elements in a polluted lake in India. Front. Microbiol. **5**.
2. **Cardoso de Oliveira AJF**, **Watanabe Pinhata JM**, **Pinhata JMW**. 2008. Antimicrobial resistance and species composition of Enterococcus spp. isolated from waters and sands of marine recreational beaches in Southeastern Brazil. Water Res. **42**:2242–2250.
3. **Cardoso de Oliveira AJF**, **Ranzani de França PT**, **Pinto AB**. 2009. Antimicrobial resistance of heterotrophic marine bacteria isolated from seawater and sands of recreational beaches with different organic pollution levels in southeastern Brazil: evidences of resistance dissemination. Environ. Monit. Assess. **169**:375–84.
4. **Marti E**, **Variatza E**, **Balcazar JL**. 2014. The role of aquatic ecosystems as reservoirs of antibiotic resistance. Trends Microbiol. **22**:36–41.
5. **Baquero F**, **Martínez JL**, **Cantón R**. 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. **19**:260–265.
6. **Alves MS**, **Pereira A**, **Araújo SM**, **Castro BB**, **Correia ACM**, **Henriques I**. 2014. Seawater is a reservoir of multi-resistant Escherichia coli, including strains hosting plasmid-mediated quinolones resistance and extended-spectrum beta-lactamases genes. Front. Microbiol. **5**:1–10.
7. **Balcázar JL**, **Subirats J**, **Borrego CM**. 2015. The role of biofilms as environmental reservoirs of antibiotic resistance. Front. Microbiol. **6**:1–9.
8. **Allen HK**, **Donato J**, **Wang HH**, **Cloud-Hansen KA**, **Davies J**, **Handelsman J**. 2010. Call of the wild: antibiotic resistance genes in natural environments. Nat. Rev. Microbiol. Rev. **8**:251–259.
9. **Miranda CD**, **Rojas R**, **Garrido M**, **Geisse J**, **González G**. 2013. Role of shellfish hatchery as a reservoir of antimicrobial resistant bacteria. Mar. Pollut. Bull. **74**:334–343.
10. **Rose JM**, **Gast RJ**, **Bogomolni A**, **Ellis JC**, **Lentell BJ**, **Touhey K**, **Moore M**. 2009. Occurrence and patterns of antibiotic resistance in vertebrates off the Northeastern United States coast. FEMS Microbiol. Ecol. **67**:421–31.
11. **Poirel L**, **Liard A**, **Nordmann P**, **Mammeri H**. 2005. Origin of Plasmid-Mediated Quinolone Resistance Determinant QnrA Origin of Plasmid-Mediated Quinolone Resistance Determinant QnrA **49**:3523–3525.
12. **Hatosy SM**, **Martiny AC**. 2015. The ocean as a global reservoir of antibiotic resistance genes. Appl. Environ. Microbiol. **81**:7593–7599.
13. **Trinh P**, **Zaneveld JR**, **Safranek S**, **Rabinowitz PM**. 2018. One Health Relationships Between Human, Animal, and Environmental Microbiomes: A Mini-Review. Front. Public Heal. **6**:1–9.
14. **Rousham EK**, **Unicomb L**, **Islam MA**. 2018. Human, animal and environmental contributors to antibiotic resistance in low-resource settings: Integrating behavioural, epidemiological and one health approaches. Proc. R. Soc. B Biol. Sci. **285**.
15. **Davies, J, & Davies, D.**, **Davies J**, **Davies D**. 2010. Origins and Evolution of Antibiotic Resistance. Microbiol. Mol. Biol. Rev. **74**:417–433.

16. **Martinez JL**, **Sánchez MB**, **Martínez-Solano L**, **Hernandez A**, **Garmendia L**, **Fajardo A**, **Alvarez-Ortega C**. 2009. Functional role of bacterial multidrug efflux pumps in microbial natural ecosystems. FEMS Microbiol. Rev. **33**:430–449.
17. **Jobbins SE**, **Alexander KA**. 2015. From Whence They Came- Antibiotic-Resistant *Escherichia coli* in African Wildlife. J. Wildl. Dis. **51**:811–820.
18. **Brandt KK**, **Amézquita A**, **Backhaus T**, **Boxall A**, **Coors A**, **Heberer T**, **Lawrence JR**, **Lazorchak J**, **Schönfeld J**, **Snape JR**, **Zhu YG**, **Topp E**. 2015. Ecotoxicological assessment of antibiotics: A call for improved consideration of microorganisms. Environ. Int. **85**:189–205.
19. **Stedtfeld RD**, **Williams MR**, **Fakher U**, **Johnson TA**, **Stedtfeld TM**, **Wang F**, **Khalife WT**, **Hughes M**, **Etchebarne BE**, **Tiedje JM**, **Hashsham SA**. 2016. Antimicrobial resistance dashboard application for mapping environmental occurrence and resistant pathogens 1–9.
20. **Berendonk TU**, **Manaia CM**, **Merlin C**, **Fatta-Kassinos D**, **Cytryn E**, **Walsh F**, **Bürgmann H**, **Sørum H**, **Norström M**, **Pons M-N**, **Kreuzinger N**, **Huovinen P**, **Stefani S**, **Schwartz T**, **Kisand V**, **Baquero F**, **Martinez JL**. 2015. Tackling antibiotic resistance: the environmental framework. Nat. Rev. Microbiol. 1–8.
21. **Bengtsson-Palme J**, **Larsson DGJ**. 2015. Antibiotic resistance genes in the environment: prioritizing risks. Nat. Rev. Microbiol. **13**:396–396.
22. **Martínez JL**, **Coque TM**, **Baquero F**. 2015. Prioritizing risks of antibiotic resistance genes in all metagenomes. Nat. Rev. Microbiol. **13**:396–396.
23. **Martinez JL**, **Coque TM**, **Baquero F**. 2015. What is a resistance gene? Ranking risk in resistomes. Nat. Rev. Microbiol. **13**:116–123.
24. **O'Neill J**. 2016. Tackling Drug-Resistant Infections Globally: Final Report and Recommendations.
25. **de Kraker MEA**, **Stewardson AJ**, **Harbarth S**. 2016. Will 10 Million People Die a Year due to Antimicrobial Resistance by 2050? PLoS Med. **13**:1–6.
26. **World Health Organization**. 2014. Antimicrobial Resistance: Global Report on surveillance.
27. **Center for Disease Control and Prevention**. 2013. Antibiotic resistance threats in the United States, 2013.
28. **Obama B**. 2014. Executive Order- Combating Antibiotic Resistant Bacteria. Washington D.C.
29. **President's Council of Advisors on Science and Technology**. 2014. Report to the President on Combating Antibiotic Resistance.
30. **Richmond EK**, **Rosi EJ**, **Walters DM**, **Fick J**, **Hamilton SK**, **Brodin T**, **Sundelin A**, **Grace MR**. 2018. A diverse suite of pharmaceuticals contaminates stream and riparian food webs. Nat. Commun. **9**:4491.
31. **Sanderson CE**, **Fox JT**, **Dougherty ER**, **Cameron ADS**, **Alexander KA**, **Alexander KA**. 2018. The Changing Face of Water : A Dynamic Reflection of Antibiotic Resistance Across Landscapes **9**:1–13.
32. **Collignon P**, **Beggs JJ**, **Walsh TR**, **Gandra S**, **Laxminarayan R**. 2018. Articles Anthropological and socioeconomic factors contributing to global antimicrobial resistance : a univariate and multivariable analysis Articles Anthropological and

socioeconomic factors contributing to global antimicrobial resistance : a univariate **2**:1–9.

33. **Vezzulli L**, **Grande C**, **Reid PC**, **Hélaouët P**, **Edwards M**, **Höfle MG**, **Brettar I**. 2016. Climate influence on *Vibrio* and associated human diseases during the past half-century in the coastal North Atlantic. Proc. Natl. Acad. Sci. **113**.

Appendix A: Open ocean samples structure resistance by biome

INTRODUCTION

Antibiotics have long existed in nature (1) before humans discovered their capabilities and began exploiting these chemicals for their own therapeutic uses. Despite this knowledge, antibiotics and subsequently, antibiotic resistance are often understudied in natural environments as researchers focus on clinical resistance in order to better inform patient management. While this makes sense in regards to patient management, it ignores the interconnected nature of humans to animals and the natural environment (e.g. the One Health concept, which seeks to study the connections between human health, animal health, and environmental health). Therefore, the natural environment provides an interesting study region to assess environmental health. Examining natural environments also allows assessment of basic scientific questions about antibiotics and antibiotic resistance such as prevalence of resistance in regions less impacted by humans, which allows for better assessment about the evolution of antibiotics/resistance and uses of these beyond cellular death.

To gain insight into these questions, the ocean was used as a study site for this chapter. The ocean is an area that humans readily interact with through food consumption and through recreation and is also affected by anthropogenic impacts (2–4). Chapters 3-5 in this thesis have illustrated that resistance is prevalent in coastal regions. These results beg the question: is this resistance common for all areas of the ocean or only coastal regions that have increased proximity to anthropogenic impacts?

To answer this question, this appendix explores the TARA oceans project (5). TARA oceans was a global sampling expedition that explored all basins of the ocean

between 2009 and 2013 to explore the diversity of the marine environment at a variety of scales (spatial and organismal). This project has already yielded many discoveries about the ocean (5–13). Further, this project has spent considerable time and energy making their data accessible, allowing an increased impact. With this open access data source, the TARA oceans project allows a unique opportunity to test hypothesis without the need for five years of sampling and the necessary associated resources.

This appendix assesses the diversity of 25 surface water samples throughout the world's ocean. The hypothesis was that surface water samples would group by biome and geographic region.

METHODS

Samples

25 samples were used from the TARA oceans dataset. Information about collection, date, latitude, and longitude can be found in Table 1. All samples were surface water samples at five meters depth (14). These samples represent a wide range of ocean biomes and have been categorized by three different measures (Table 1). One is the Longhurst biomes, which groups all areas of the ocean into four main categories: polar, westerlies, trades, and coastal biomes (15, 16). Longhurst also has a more detailed categorization called provinces. Another categorization is the International Hydrographic Organization's General Sea Areas (17). All of these groupings are to better understand and analysis the ocean by its similar regions. Samples were all processed by the TARA Oceans group (14).

Data acquisition

Raw sequencing reads were downloaded from European Nucleotide Archive. Raw reads were then uploaded into MetaStorm, an online server to process metagenomic data (18). This server was also used in Chapter 5 and more details about it can be read there. However, for this appendix, only the assembly pipeline was used.

Statistics

As in chapter 5, permutational multivariate analysis of variance (PERMANOVA) was utilized in Primer (version 7) (19). For this data, normalized gene abundance for each database was tested across the samples using a one way PERMANOVA for each biome categorization- Longhurst marine biomes, Longhurst marine provinces, and IHO ocean and sea regions. The same tests were also run on normalized prevalence of all genes for each database.

To compare coastal and open ocean samples from the TARA data, normalized gene abundance was tested using one way PERMANOVAs.

RESULTS

Metagenomic sampling

Most samples had 20-40 million raw read, though there were a few samples that had significantly less reads at 7 million raw reads (Table 2). A little less than half of the reads were able to be assembled. ACLAME, the mobile genetic element database had the highest percentage of genes annotated to it between 0-4%. BacMet followed with 0-0.13%, then CARD and ARDB. CARD had substantially more database hits than ARDB.

Top Genes

The top twenty genes of each grouping or database were determined. ACLAME showed a variety of plasmid families relating to oxidoreductase activity and ATP binding cassettes. BacMet showed a diversity of genes focusing on many metals, but the top spot was for copper. ARDB's top 20 ARG included many different antibiotics. 11/20 of the top 20 genes in ARDB are efflux genes and 15/ 20 were efflux genes in the CARD samples. Genes shared between both databases were mexF and mexB.

PERMANOVA results

All tests of gene abundance by database for each biome were significant, except for CARD. For tests looking at gene prevalence among samples, almost all tests were significant. The one insignificant test is CARD for the Longhurst 2007. An nMDS plot shows data for BacMet grouping primarily by biome, in this case Longhurst province (Figure 1). Finally, for the comparisons between coastal and open ocean samples, gene abundance was significant for all databases.

DISCUSSION

This study represents one of the first, to our knowledge, to elucidate what resistance genes are present in the open ocean.

Gene recovery

In all but two samples, ARG were found for both CARD and ARDB. The two samples that did not have ARG actually appeared to have other issues within the metagenomic analysis and perhaps should not be included. In general, CARD had a higher number of hits compared to ARDB, which is the same as what was seen for Chapter 5 with the coastal samples.

Gene abundance significance

Gene abundance was significant for all databases, but CARD. This indicates that amounts of genes are significantly different between these biomes. Further research would be necessary to determine what differences are found and between which samples.

Biogeography dynamics

The significance between biomes indicates that areas of the ocean are distinct from each other, based on their biome grouping for mobile genetic elements, antibiotic and metal resistance genes. It also indicates that samples are relatively stable in these regions and groupings because these areas are distinct even though samples were taken at different time periods. Grouping by Longhurst province can be seen in Figure 1. This indicates that studying the evolutionary aspects of resistance in the open ocean has interesting and compelling potential.

Comparison between coastal and TARA samples

There were a remarkable number of similarities in the top 20 ACLAME families between coastal and TARA with 13 shared families (1, 6, 5648, 321, 26, 11, 53,9,205,64, 48, 10905, 665). These represent groupings that are avenues to more deeply examine why they may be prevalent globally and what affects they may have on resistance. For BacMet, different top genes were shown and the top group for coastal metagenomes ("Other") was not even on the list for the TARA samples, indicating some differences in abundance here. Perhaps the other category includes more prevalent anthropogenic materials that are less prevalent in the open ocean. For the antibiotic resistance databases, there were more efflux genes in CARD for the open ocean prevalence compared to the coastal area. PERMANOVA illustrated that there are distinctions in efflux gene abundance

between the four coastal impacts in Chapter 5 (industrial, wastewater, reference, and wastewater Boston) and the four Longhurst biomes (coastal, westerlies, polar, and trades). This provides further acknowledgement that efflux genes are prevalent in the ocean and that their underlying purpose/evolutionary process would provide interesting research. It also provides the potential that coastal regions may have more specific resistance genes because of their exposures to anthropogenic activities.

Future Directions

As seen in this overview for this appendix, there are a remarkable number of leads to follow. One is examining all the available surface ocean samples for TARA, which is currently in process, but did not happen to fit into this dissertation. Next, analyzing samples with ocean depth to see if there are similarities across depths. In addition, both of these should be studied in detail to see what genes may be driving any similarities or differences.

References

1. **D'Costa VM**, **King CE**, **Kalan L**, **Morar M**, **Sung WWL**, **Schwarz C**, **Froese D**, **Zazula G**, **Calmels F**, **Debruyne R**, **Golding GB**, **Poinar HN**, **Wright GD**. 2011. Antibiotic resistance is ancient. Nature **477**:457–61.
2. **Jamieson AJ**, **Malkocs T**, **Piertney SB**, **Fujii T**, **Zhang Z**. 2017. Bioaccumulation of persistent organic pollutants in the deepest ocean fauna. Nat. Ecol. Evol. **1**:24–27.
3. **Morét-Ferguson S**, **Law KL**, **Proskurowski G**, **Murphy EK**, **Peacock EE**, **Reddy CM**. 2010. The size, mass, and composition of plastic debris in the western North Atlantic Ocean. Mar. Pollut. Bull. **60**:1873–8.
4. **Law KL**, **Thompson RC**. 2014. Oceans. Microplastics in the seas. Science **345**:144–5.
5. **Sunagawa S**, **Coelho LP**, **Chaffron S**, **Kultima JR**, **Labadie K**, **Salazar G**, **Djahanschiri B**, **Zeller G**, **Mende DR**, **Alberti A**, **Cornejo-Castillo FM**, **Costea PI**, **Cruaud C**, **D'Ovidio F**, **Engelen S**, **Ferrera I**, **Gasol JM**, **Guidi L**, **Hildebrand F**, **Kokoszka F**, **Lepoivre C**, **Lima-Mendez G**, **Poulain J**, **Poulos BT**, **Royo-Llonch M**, **Sarmento H**, **Vieira-Silva S**, **Dimier C**, **Picheral M**, **Searson S**, **Kandels-Lewis S**, **Bowler C**, **de Vargas C**, **Gorsky G**, **Grimsley N**, **Hingamp P**, **Iudicone D**, **Jaillon O**, **Not F**, **Ogata H**, **Pesant S**, **Speich S**, **Stemmann L**, **Sullivan MB**, **Weissenbach J**, **Wincker P**, **Karsenti E**, **Raes J**, **Acinas SG**, **Bork P**. 2015. Ocean plankton. Structure and function of the global ocean microbiome. Science **348**:1261359.
6. **Pesant S**, **Not F**, **Picheral M**, **Kandels-Lewis S**, **Le Bescot N**, **Gorsky G**, **Iudicone D**, **Karsenti E**, **Speich S**, **Trouble R**, **Dimier C**, **Searson S**. 2015. Open science resources for the discovery and analysis of Tara Oceans data. Sci. Data **2**:1–16.
7. **Werdell PJ**, **Proctor CW**, **Boss E**, **Leeuw T**, **Ouhssain M**. 2013. Underway sampling of marine inherent optical properties on the Tara Oceans expedition as a novel resource for ocean color satellite data product validation. Methods Oceanogr. **7**:40–51.
8. **Lima-Mendez G**, **Faust K**, **Henry N**, **Decelle J**, **Colin S**, **Carcillo F**, **Chaffron S**, **Ignacio-Espinosa JC**, **Roux S**, **Vincent F**, **Bittner L**, **Youssef D**, **Wang J**, **Audic S**,

Berline L, **Bontempi G**, **Cabello AM**, **Coppola L**, **Cornejo-Castillo FM**, **D'Ovidio F**, **de Meester L**, **Ferrera I**, **Garet-Delmas M-J**, **Guidi L**, **Lara E**, **Pesant S**, **Royo-Llonch M**, **Salazar G**, **Sánchez P**, **Sebastian M**, **Souffreau C**, **Dimier C**, **Picheral M**, **Searson S**, **Kandel-Lewis S**, **Tara Oceans Coordinators**, **Gorsky G**, **Not F**, **Ogata H**, **Speich S**, **Stemmann L**, **Weissenbach J**, **Wincker P**, **Acinas SG**, **Sunagawa S**, **Bork P**, **Sullivan MB**, **Karsenti E**, **Bowler C**, **de Vargas C**, **Raes J**. 2015. Determinants of community structure in the global plankton interactome. Science (80-.). **348**:1262073_1-1262073_9.

9. **Brum JR**, **Ignacio-espinoza JC**, **Roux S**, **Doulcier G**, **Acinas SG**, **Alberti A**, **Chaffron S**. 2015. Ocean Viral Communities. Science (80-.). **348**:1261498-1–11.
10. **Logares R**, **Sunagawa S**, **Salazar G**, **Cornejo-Castillo FM**, **Ferrera I**, **Sarmento H**, **Hingamp P**, **Ogata H**, **de Vargas C**, **Lima-Mendez G**, **Raes J**, **Poulain J**, **Jaillon O**, **Wincker P**, **Kandels-Lewis S**, **Karsenti E**, **Bork P**, **Acinas SG**. 2013. Metagenomic 16S rDNA Illumina tags are a powerful alternative to amplicon sequencing to explore diversity and structure of microbial communities. Environ. Microbiol.
11. **de Vargas C**, **Audic S**, **Henry N**, **Decelle J**, **Mahé F**, **Logares R**, **Lara E**, **Berney C**, **Le Bescot N**, **Probert I**, **Carmichael M**, **Poulain J**, **Romac S**, **Colin S**, **Aury J-M**, **Bittner L**, **Chaffron S**, **Dunthorn M**, **Engelen S**, **Flegontova O**, **Guidi L**, **Horák A**, **Jaillon O**, **Lima-Mendez G**, **Lukeš J**, **Malviya S**, **Morard R**, **Mulot M**, **Scalco E**, **Siano R**, **Vincent F**, **Zingone A**, **Dimier C**, **Picheral M**, **Searson S**, **Kandels-Lewis S**, **Acinas SG**, **Bork P**, **Bowler C**, **Gorsky G**, **Grimsley N**, **Hingamp P**, **Iudicone D**, **Not F**, **Ogata H**, **Pesant S**, **Raes J**, **Sieracki ME**, **Speich S**, **Stemmann L**, **Sunagawa S**, **Weissenbach J**, **Wincker P**, **Karsenti E**. 2015. Ocean plankton. Eukaryotic plankton diversity in the sunlit ocean. Science **348**:1261605.
12. **Malviya S**, **Scalco E**, **Audic S**, **Vincent F**, **Veluchamy A**, **Poulain J**, **Wincker P**, **Iudicone D**, **de Vargas C**, **Bittner L**, **Zingone A**, **Bowler C**. 2016. Insights into global diatom distribution and diversity in the world's ocean. Proc. Natl. Acad. Sci. **113**:E1516–E1525.
13. **Lescot M**, **Hingamp P**, **Kojima KK**, **Villar E**, **Romac S**, **Veluchamy A**, **Boccara M**,

Jaillon O, **Iudicone D**, **Bowler C**, **Wincker P**, **Claverie JM**, **Ogata H**. 2016. Reverse transcriptase genes are highly abundant and transcriptionally active in marine plankton assemblages. ISME J. **10**:1134–1146.

14. **Sunagawa S**, **Coelho LP**, **Chaffron S**, **Kultima JR**, **Labadie K**, **Salazar G**, **Djahanschiri B**, **Zeller G**, **Mende DR**, **Alberti A**, **Cornejo-castillo FM**, **Costea PI**, **Cruaud C**, **Ovidio F**, **Engelen S**, **Ferrera I**, **Gasol JM**, **Guidi L**, **Hildebrand F**, **Kokoszka F**, **Lepoivre C**. 2015. Structure and function of the global ocean microbiome. Science (80-.). **348**:1–9.
15. **Longhurst A**. 2007. Ecological Geography of the SeaSecond. Academic Press.
16. **Miller CB**, **Wheeler PA**. 2012. Biological OceanographySecond. Wiley & Sons, West Sussex.
17. **International Hydrographic Organization**. 2014. Internation Hydrographic Organization.
18. **Arango-Argoty G**, **Singh G**, **Heath LS**, **Pruden A**, **Xiao W**, **Zhang L**. 2016. MetaStorm : A Public Resource for Customizable Metagenomics Annotation 1–13.
19. **Clarke KR**, **Gorley RN**. 2015. PRIMER v7 : User Manual and Tutorial. Plymouth.

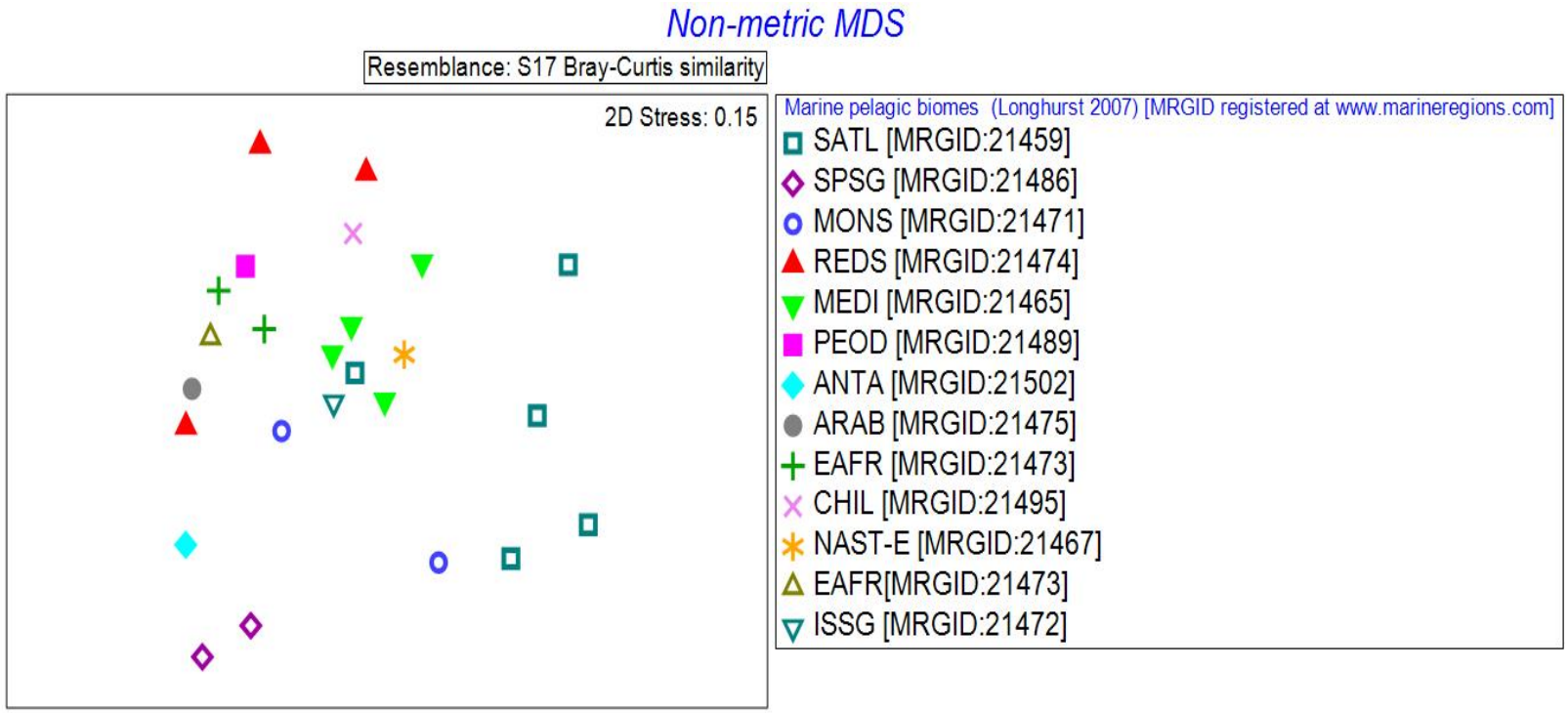


Figure 1. nMDS plot of 25 TARA samples data.
Data is categorized with the BacMet data using the Longhurst detailed marine biomes

Table 1. TARA metagenomic sample information.
This table illustrates the samples used for this appendix with location and oceanic biome categorization. Information is from the TARA project (14).

Sample ID	Tara Station	Date/Time [yyyy-mm-ddThh:mm]	Latitude [degrees North]	Longitude [degrees East]	depth [m]	Size fraction lower threshold [um]	Size fraction upper threshold [um]	Longhurst marine biomes	Longhurst marine provinces[2]	IHO General Sea Areas [3]
ERR315858 TARA_023_ SRF_0.22-1.6	23	2009-11-18T08:41	42.2038	17.715	5	0.22	1.6	Westerlies	MEDI	MS
ERR315861 TARA_023_ SRF_0.22-1.6	23	2009-11-18T08:41	42.2038	17.715	5	0.22	1.6	Westerlies	MEDI	MS
ERR594317 TARA_009_ SRF_0.22-1.6	9	2009-09-28T12:18	39.1633	5.916	5	0.22	1.6	Westerlies	MEDI	MS
ERR598943 TARA_102_ SRF_0.22-3	102	2011-04-21T20:07	-5.2529	-85.1545	5	0.22	3	Trades	PEOD	SPO
ERR598945 TARA_084_ SRF_0.22-3	84	2011-01-03T11:05	-60.2287	-60.6476	5	0.22	3	Polar	ANTA	SO

[2] Abbreviations for Longhurst marine provinces: (ANTA) Antarctic Province, (ARAB) Northwest Arabian Sea Upwelling Province, (CHIL) Chile-Peru Current Coastal Province, (EAFR) Eastern Africa Coastal Province, (ISSG) Indian South Subtropical Gyre Province, (MEDI) Mediterranean Sea, Black Sea Province, (MONS) Indian Monsoon Gyres Province, (NAST-E) North Atlantic Subtropical Gyral Province, (PEOD) Pacific Equatorial Divergence Province, (REDS) Red Sea, Persian Gulf Province, (SATL) South Atlantic Gyral Province, (SPSG) South Pacific Subtropical Gyre Province, North and South

[3] Abbreviations for International Hydrographic Organization (IHO) ocean and sea regions IO: Indian Ocean, MS: Mediterranean Sea, NPO: North Pacific Ocean, RS: Red Sea, SAO: Southern Atlantic Ocean, SO: Southern Ocean, SPO: Southern Pacific Ocean

ERR598951 TARA_025_ SRF_0.22-1.6	25	2009-11-23T09:12	39.3888	19.3905	5	0.22	1.6	Westerlies	MEDI	MS
ERR598959 TARA_034_ SRF_0.22-1.6	34	2010-01-20T04:27	18.3967	39.875	5	0.22	1.6	Coastal	REDS	RS
ERR598966 TARA_036_ SRF_0.22-1.6	36	2010-03-12T06:06	20.8183	63.5047	5	0.22	1.6	Coastal	ARAB	IO
ERR598969 TARA_031_ SRF_0.22-1.6	31	2010-01-09T07:15	27.16	34.835	5	0.22	1.6	Coastal	REDS	RS
ERR598970 TARA_064_ SRF_0.22-3	64	2010-07-07T04:48	-29.5019	37.9889	5	0.22	3	Coastal	EAFR	IO
ERR598979 TARA_065_ SRF_0.22-3	65	2010-07-12T05:59	-35.1728	26.2868	5	0.22	3	Coastal	EAFR	IO
ERR598997 TARA_109_ SRF_0.22-3	109	2011-05-12T14:00	1.9928	-84.5766	5	0.22	3	Coastal	CHIL	NPO
ERR599003 TARA_004_ SRF_0.22-1.6	4	2009-09-15T11:30	36.5533	-6.5669	5	0.22	1.6	Westerlies	NAST-E	NAO
ERR599010 TARA_076_ SRF_0.22-3	76	2010-10-16T09:55	-20.9354	-35.1803	5	0.22	3	Trades	SATL	SAO
ERR599022 TARA_078_	78	2010-11-04T10:04	-30.1367	-43.2899	5	0.22	3	Trades	SATL	SAO

SRF_0.22-3										
ERR599088 TARA_064_ SRF_0.22-3	64	2010-07-07T04:48	-29.5019	37.9889	5	0.22	3	Coastal	EAFR	IO
ERR599098 TARA_052_ SRF_0.22-1.6	52	2010-05-17T04:10	-16.957	53.9801	5	0.22	1.6	Trades	ISSG	IO
ERR599105 TARA_072_ SRF_0.22-3	72	2010-10-05T08:00	-8.7789	-17.9099	5	0.22	3	Trades	SATL	SAO
ERR599114 TARA_125_ SRF_0.22-3	125	2011-08-08T17:33	-8.9111	-142.5571	5	0.22	3	Trades	SPSG	SPO]
ERR599119 TARA_125_ SRF_0.22-3	125	2011-08-08T17:33	-8.9111	-142.5571	5	0.22	3	Trades	SPSG	SPO
ERR599135 TARA_070_ SRF_0.22-3	70	2010-09-21T06:55	-20.4091	-3.1759	5	0.22	3	Trades	SATL	SAO
ERR599141 TARA_042_ SRF_0.22-1.6	42	2010-04-04T02:47	6.0001	73.8955	5	0.22	1.6	Trades	MONS	IO
ERR599155 TARA_032_ SRF_0.22-1.6	32	2010-01-11T07:21	23.36	37.2183	5	0.22	1.6	Coastal	REDS	RS
ERR599158 TARA_038_ SRF_0.22-1.6	38	2010-03-15T03:35	19.0393	64.4913	5	0.22	1.6	Trades	MONS	IO

ERR599171 TARA_068_ SRF_0.22-3	68	2010-09-14T06:55	-31.0266	4.665	5	0.22	3	Trades	SATL	SAO

Table 2. Metagenomics summary statistics of TARA ocean samples.

Sample ID	Raw Reads	Input Reads after QC	Assembled reads (#)	Assembled reads (%)	Scaffolds	Average scaffold length (bps)	N50 of scaffolds (bps)	Total predicted genes	Average gene length (bps)
ERR315858 TARA_023_SRF_0.22-1.6	37,472,639	34,182,891	16,559,385	48.44	568,091	752	903	814,908	481
ERR315861 TARA_023_SRF_0.22-1.6	37,310,366	34,641,768	16,847,647	48.63	581,069	750	898	831,320	481
ERR594317 TARA_009_SRF_0.22-1.6	72,867,764	70,801,473	34,471,816	48.69	608,841	903	1,264	994,513	506
ERR598943 TARA_102_SRF_0.22-3	36,334,542	33,347,889	14,657,180	43.95	503,857	730	803	754,203	453
ERR598945 TARA_084_SRF_0.22-3	33,639,827	33,146,520	15,362,545	46.35	372,645	688	717	501,024	436
ERR598951 TARA_025_SRF_0.22-1.6	30,171,447	25,956,492	12,369,958	47.66	374,720	785	928	584,442	461
ERR598959 TARA_034_SRF_0.22-1.6	39,588,227	35,693,819	17,780,301	49.81	425,517	791	939	651,012	477
ERR598966 TARA_036_SRF_0.22-1.6	43,069,804	39,554,266	20,340,871	51.43	461,948	738	834	705,798	449
ERR598969 TARA_031_SRF_0.22-1.6	34,486,935	28,632,499	10,341,392	36.12	351,635	652	681	494,871	426
ERR598970 TARA_064_SRF_0.22-3	42,483,957	35,174,262	11,921,822	33.89	442,596	613	641	598,568	417
ERR598979 TARA_065_SRF_0.22-3	47,194,699	31,671,906	11,294,401	35.66	364,330	707	760	529,558	449
ERR598997 TARA_109_SRF_0.22-3	26,789,701	24,707,354	9,864,950	39.93	315,629	706	760	461,401	448
ERR599003 TARA_004_SRF_0.22-1.6	28,822,442	24,126,231	10,337,916	42.85	328,016	718	777	489,051	445
ERR599010 TARA_076_SRF_0.22-3	30,059,067	20,001,993	3,143,478	15.72	179,691	548	539	230,211	367
ERR599022	31,241,187	26,048,500	6,456,093	24.78	275,739	616	628	376,169	400

TARA_078_SRF_0.22-3									
ERR599088 TARA_064_SRF_0.22-3	41,574,000	34,535,732	11,650,309	33.73	432,251	612	642	584,307	417
ERR599098 TARA_052_SRF_0.22-1.6	29,188,354	27,026,575	11,091,963	41.04	330,478	711	774	489,789	443
ERR599105 TARA_072_SRF_0.22-3	34,374,500	29,896,086	7,830,010	26.19	347,212	597	597	489,626	387
ERR599114 TARA_125_SRF_0.22-3	31,492,000	31,073,278	14,372,530	46.25	435,122	728	793	641,675	459
ERR599119 TARA_125_SRF_0.22-3	41,818,200	41,239,998	19,741,818	47.87	565,216	730	796	831,787	460
ERR599135 TARA_070_SRF_0.22-3	7,588,000	7,365,300	884,282	12.01	87,941	502	476	105,102	363
ERR599141 TARA_042_SRF_0.22-1.6	36,435,982	30,438,279	12,711,905	41.76	380,677	687	749	563,061	431
ERR599155 TARA_032_SRF_0.22-1.6	22,345,279	19,414,565	7,200,570	37.09	204,321	665	694	298,991	419
ERR599158 TARA_038_SRF_0.22-1.6	39,699,921	34,587,590	14,834,066	42.89	407,687	666	694	597,738	420
ERR599169 TARA_100_SRF_0.22-3	14,532,000	14,242,668	36,714	0.26	8	36,013	34,959	281	867
ERR599171 TARA_068_SRF_0.22-3	36,508,689	9,403,028	1,092,485	11.62	91,303	511	493	111,955	366
ERR599176 TARA_085_SRF_0.22-3	37,811,950	37,323,096	48,802	0.13	5	50,294	49,047	241	963

Table 3. Gene recovery information for each database.

Sample ID	Total genes	ACLAME genes	% ACLAME	BacMet Genes	% BacMet	CARD genes	% CARD	ARDB genes	% ARDB
ERR315858 TARA_023_SRF_0.22-1.6	814,908	35,548	4.36	1098	0.13	575	0.07	62	0.01
ERR315861 TARA_023_SRF_0.22-1.6	831,320	35,792	4.31	1,130	0.14	595	0.07	64	0.01
ERR594317 TARA_009_SRF_0.22-1.6	994,513	34,214	3.44	1082	0.11	539	0.05	80	0.01
ERR598943 TARA_102_SRF_0.22-3	754,203	21,133	2.80	637	0.08	341	0.05	20	0.00
ERR598945 TARA_084_SRF_0.22-3	501,024	15,852	3.16	526	0.10	212	0.04	28	0.01
ERR598951 TARA_025_SRF_0.22-1.6	584,442	22,651	3.88	689	0.12	319	0.05	44	0.01
ERR598959 TARA_034_SRF_0.22-1.6	651,012	18,778	2.88	559	0.09	297	0.05	15	0.00
ERR598966 TARA_036_SRF_0.22-1.6	705,798	21,445	3.04	519	0.07	315	0.04	14	0.00
ERR598969 TARA_031_SRF_0.22-1.6	494,871	17,295	3.49	474	0.10	241	0.05	15	0.00
ERR598970 TARA_064_SRF_0.22-3	598,568	19,307	3.23	587	0.10	335	0.06	17	0.00
ERR598979 TARA_065_SRF_0.22-3	529,558	15,346	2.90	415	0.08	214	0.04	10	0.00
ERR598997 TARA_109_SRF_0.22-3	461,401	15,442	3.35	428	0.09	244	0.05	16	0.00
ERR599003 TARA_004_SRF_0.22-1.6	489,051	19,337	3.95	513	0.10	277	0.06	41	0.01
ERR599010	230,211	7,384	3.21	261	0.11	115	0.05	16	0.01

TARA_076_SRF_0.22-3									
ERR599022 TARA_078_SRF_0.22-3	376,169	13,443	3.57	371	0.10	186	0.05	25	0.01
ERR599088 TARA_064_SRF_0.22-3	584,307	19057	3.26	551	0.09	332	0.06	15	0.00
ERR599098 TARA_052_SRF_0.22-1.6	489,789	15,331	3.13	421	0.09	259	0.05	22	0.00
ERR599105 TARA_072_SRF_0.22-3	489,626	12,288	2.51	475	0.10	188	0.04	39	0.01
ERR599114 TARA_125_SRF_0.22-3	641,675	22,272	3.47	750	0.12	412	0.06	28	0.00
ERR599119 TARA_125_SRF_0.22-3	831,787	28,601	3.44	1074	0.13	548	0.07	37	0.00
ERR599135 TARA_070_SRF_0.22-3	105,102	2725	2.59	113	0.11	38	0.04	9	0.01
ERR599141 TARA_042_SRF_0.22-1.6	563,061	17,879	3.18	586	0.10	321	0.06	37	0.01
ERR599155 TARA_032_SRF_0.22-1.6	298,991	8814	2.95	251	0.08	146	0.05	9	0.00
ERR599158 TARA_038_SRF_0.22-1.6	597,738	15,489	2.59	428	0.07	248	0.04	27	0.00
ERR599169 TARA_100_SRF_0.22-3	281	1	0.36	0	0.00	0	0.00	0	0.00
ERR599171 TARA_068_SRF_0.22-3	111,955	2806	2.51	105	0.09	52	0.05	16	0.01
ERR599176 TARA_085_SRF_0.22-3	241	0	0.00	0	0.00	0	0.00	0	0.00