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Antibiotic Resistance of Bacterial Communities from the Los Angeles River to the Pacific Ocean

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Antibiotic Resistance of Bacterial Communities from the Los Angeles River to the Pacific Ocean

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology

by

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ABSTRACT OF THE THESIS

Antibiotic Resistance of Bacterial Communities from the Los Angeles River to the Pacific Ocean

by

Carolyn Xue

Master of Science in Biology

University of California, Los Angeles, 2023

Professor Pamela J. Yeh, Chair

Widespread overuse and large-scale production of antibiotics create antibiotic pollution, disrupting environmental microbiota and creating a public health risk. Highly urbanized coastal environments can be under high impact from antibiotic pollution from many trails of polluted effluents and runoff. The city of Long Beach is one such coastal area under high impact, since it is highly urban, industrialized, and experiences frequent sewage spills. We collected water samples from the LA River in a transect running southwest into the San Pedro Channel in order to investigate (1) how does strength of antibiotic resistance change as distance from shore increases, and (2) does antibiotic resistance correlate with composition of the bacterial community. There was no consistent relationship between strength of antibiotic resistance and distance from shore. Instead, we found that bacteria from the Pacific Ocean showed higher antibiotic resistance than bacteria from the LA River in five out of eleven antibiotic treatments. We also found that the alpha diversity of bacterial communities was lower in the LA River samples compared, and alpha diversity positively correlated with strength of antibiotic resistance in four antibiotic treatments. Our findings highlight how prevalence of antibiotic pollution does not always follow a distance dilution, as well as the need for understanding the strength behind antibiotic resistance in marine bacteria.

The thesis of Carolyn Xue is approved.

Michael Edward Alfaro

Jennifer Ayla Jay

Pamela J. Yeh, Committee Chair

University California, Los Angeles

2023

Dedicated to my grandfather.

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ACKNOWLEDGEMENTS

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Introduction

The global spread of antibiotic resistant bacteria is a public health concern that is driven by urbanization and changes to environmental microbiomes[1,2]. Sources of antibiotic pollution begin wherever there is large-scale use and production of antibiotics, in clinical, industrial, and agricultural settings[3]. Some common forms of polluted water that facilitate movement of antibiotic resistant bacteria and their genes are via waste streams from hospitals and industrial sources of antibiotic production, runoff from agricultural feeding operations and aquaculture, and excrement from humans and domestic animals[1,3]. Wastewater treatment plants that manage effluents from urban sources have several methods for removing antibiotic resistant bacteria from the water, but all with varying success[4]. Increased use and industrial production of antibiotics since the 1940's have been correlated with an increased 'background' abundance and diversity of antibiotic resistance genes in the environment, seen in the microbiomes of the soil[5], water[6], and wildlife[7]. Once new resistance genotypes have evolved, they are highly persistent in the environmental reservoir of antibiotic resistance genes[8,9]. Trace concentrations of antibiotic compounds can be enough to select for resistance, and have been detected in environments in close proximity to anthropogenic sources[10].

Highly urbanized coastal environments can act as an eventual sink for antibiotic resistant bacteria through the many trails of polluted effluents or runoff. Although the risk of acquiring a resistant infection from environmental bacteria is unclear in terrestrial environments[1,11], coastal environments have shown more direct evidence of altering the microbiome of people, posing a public health risk. The skin microbiomes of people who have swum in the ocean showed higher antibiotic resistance gene abundance up to 6 hours later[12]. A survey of British surfers found that they were three times more likely to harbor strains of antibiotic resistant *E. coli* within their gut

microbiota compared to people not exposed to coastal waters[13]. In a separate study, swimming in the ocean was found to be a risk factor for urinary tract infections caused by *E. coli*[14]. Considering that 37% of the world's population lives within 100 km of a coast[15], coastal environments are under high impact from pollution and disruption of environmental microbiomes.

Certain characteristics of the marine environment make it conducive to the spread of antibiotic resistance. Metagenomic analysis reveals that, while the marine environment holds a great diversity of antibiotic resistance genes, the majority of resistance genes found were never previously described[16]. Antibiotic resistance genes were also harbored in marine bacteria that are abundant in the ocean, such as those from the orders of Rhodobacterales and Roseobacter, suggesting that the ocean could be a massive reservoir for antibiotic resistance[16]. Furthermore, marine bacteria can facilitate horizontal gene transfer through production of gene transfer agents. This mechanism is similar to transduction in horizontal gene transfer, but involves the bacteria spontaneously producing virus-like particles and packaging their own pieces of DNA into them[17,18]. Such a mechanism, observed in Rhodobacterales and Roseobacter, implies that marine bacteria are capable of horizontal gene transfer at a frequency several thousand times faster than what have been previously measured in marine environments[19]. A characteristic of marine bacterial communities may therefore be greater fluidity in genetic exchange and greater adaptability in response to environmental changes[18].

The city of Long Beach is a highly urban coastal city, whose polluted waters have posed a public health burden. With a population of 466,742, it is the second largest city of Los Angeles County[20]. Long Beach is highly industrialized, being one of the largest shipping container ports in the United States, while also having several recreational beaches. The LA River experiences

frequent sewage spills, which affects the downstream waters of Long Beach and have forced beach closures. Five massive sewage spills in the LA River have occurred in the past two years; 17 million gallons were spilled in July 2021[21], 4 million gallons in December 2021[22], 12,000 gallons in February 2022[23], 30-40,000 gallons in March 2022[24], and 250,000 gallons in April 2023[25]. As the coastal areas of Long Beach are under high impact, we searched for patterns of how antibiotic resistant bacteria from anthropogenic sources affect the marine environment at large.

In this study, we aimed to compare the bacterial community composition and strength of antibiotic resistance in water collected from the LA River and the Pacific Ocean. Samples from the Pacific Ocean were collected in a transect for several miles southwest from the Port of Long Beach. We performed a metagenomic analysis through 16S amplicon sequencing to determine how bacterial community composition changed with increasing distance from shore. We also measured how functionally resistant the bacteria samples were by treating them with antibiotics and measuring the minimum inhibitory concentration (MIC). Our study aimed to answer the following questions about the water samples we collected: (1) how does strength of antibiotic resistance change as distance from shore increases? And (2) does antibiotic resistance correlate with composition of the bacterial community?

Methods

Sample collection

Two samples were collected from the LA River approximately 6 km inland (designated as Site 1), and the other from an estuary, where the LA River meets the Pacific Ocean in Long Beach (designated as Site 2). Six samples from the Pacific Ocean were collected approximately

every 5.5 km, with the first saltwater sample collected at the outer edge of the Port of Los Angeles (Fig. 1). These sites were named Site 3 and onwards. Ocean transects were conducted aboard the R/V Yellowfin as part of the San Pedro Ocean Timeseries[26]. The research vessel departed from the Southern California Marine Institute and traveled in a southwest transect for 24 km into the San Pedro Channel, stopping at collection sites. A collection bucket was lowered into the water and 2 L of water were collected at surface level (< 1 m). Latitude, longitude, distance from Site 2, and time of collection were recorded at each sampling site. Properties of the water were also collected: salinity, pH, and temperature (Table 1). Depending on the salinity measured, Site 1 was designated as a 'freshwater' sample and all other sites were designated as 'saltwater' samples for experimental conditions and statistical analysis.

Of the 2 L of water collected from each site, 100 mL were set aside and split into 4 replicates of 25 mL, creating 32 bacterial samples for use in experiments. These samples were mixed directly with Luria Broth (LB) media for culturing overnight. Four mL from each culture were then mixed with 50% glycerol and stored in cryotubes as frozen stock in -80 °C. The remaining 1.9 L of the collected water samples was split into 2 to create duplicates, and each 950 mL duplicate was filtered in order to concentrate bacteria for DNA extraction and sequencing. Water was filtered through a 10 μm pore diameter membrane, then filtered again through a 0.22 μm pore diameter membrane. Filtrate was discarded, and each of the 16 membranes was placed in 50 mL of fresh water or artificial seawater to resuspend the sediment that remained on the membranes. This water was then used for DNA extraction.

DNA extraction and sequencing

A Qiagen DNEasy Blood & Tissue kit was used according to manufacturer's instructions to extract DNA from bacteria from freshwater and saltwater samples. Two extractions were performed per water sample from a site, making a total of 16 DNA samples. Eluted DNA concentration was quantified using a Qubit fluorometer, and concentrations per sample ranged from 0.04 to 0.5 ug/uL. DNA samples were then shipped to the Microbial Analysis, Resources, and Services facility at the University of Connecticut for 16S rRNA Amplicon sequencing. Library preparation, alignment, and OTU classification were performed using the mothur pipeline[27].

Community composition was visualized using 'TAXONOMY' format files produced by the pipeline and visualized using Krona. OTU tables produced by the pipeline were further analyzed and visualized using the *vegan* package in R[28,29]. Shannon diversity index and Jaccard dissimilarity index were calculated for alpha and beta diversity, respectively. Indicator species, found as OTUs with the highest association to a group, were found using the *indicspecies* package in R[30].

Minimum inhibitory concentration (MIC) experiments

The frozen stocks of bacteria from each collection site, containing a subsample of the bacterial community, were thawed and placed in LB media for culturing. LB media was made with either freshwater or artificial seawater as the solvent. Approximately 20 µL of each bacterial sample would be placed in 20 mL of LB, incubated for 16-20 hours overnight, then 1:1,000 dilutions of these cultures were used for MIC experiments. 100 µL of LB media were first added to every well of 96-well culture plates. For most antibiotics, initial concentrations starting at

4,000 mg/mL were loaded onto a 96-well cell culture plate, then serially diluted by half to give 20 decreasing concentrations, ranging from 2,000 mg/mL to 0.004 mg/mL. One column of wells will receive no antibiotic treatment, designated as the positive control. 12 antibiotics were selected in order to span a variety of drug classes and mechanisms, and were also selected for their clinical relevance (Table 2). 100 μL of the diluted bacterial cultures were then added to every well, except for a column designated as the negative control. 96-well culture plates were allowed to grow overnight, then placed into a microplate reader to determine growth. The absorbance of a 96-well plate containing only antibiotic solutions, LB media, and no bacteria was also measured to subtract away background absorbance values created by the color of LB media or antibiotic solution. A total of 384 MIC experiments were performed.

Dose response curves were fitted to plate absorbance data using the *drc* package in R[31]. MICs were determined to be the antibiotic concentration that inhibited the growth of 95% of bacteria compared to positive controls. MIC values were grouped by antibiotic treatment, and a t-test was performed to compare MIC values between freshwater and saltwater samples. Pearson's correlation tests were also performed to investigate correlation between MIC values and the average Shannon diversity index of each site.

Temperature performance curve (TPC) experiments

We decided to run TPC experiments after observing high MIC values in saltwater bacteria, and noting that marine sites showed different temperatures. We hypothesized that the saltwater bacteria may be thermally adapted, since thermal adaptation in bacteria is known to also confer antibiotic resistance[32]. TPC experiments were performed to measure optimum temperatures (T_{opt}) and tolerated temperature range for growth for bacteria samples (T_{min} and

T_{max}). All 32 bacterial culture samples (4 replicates from each of the 8 sites) were thawed and placed in LB media made with freshwater or artificial seawater. Samples were allowed to culture overnight for approximately 16 hours, then diluted down by a factor of 1,000. 100 µL of each of the 32 samples were then loaded in triplicates onto a single 96-well culture plate, along with 100 µL of LB media, and 22 plate replicates were prepared using pin transfer. 22 incubators were set with a temperature within the range of 10 °C to 52 °C, increasing by increments of 2. Plate absorbances were read every 2 hours for 24 hours.

Growth curves were fitted onto absorbance data using the *growthTools* package developed by Dr. Colin Kremer[33]. Parameters in fitting exponential growth curves were then extracted to determine the T_{opt}, T_{min}, and T_{max}.

Results

We sequenced bacterial communities to understand the composition of each site. Sequencing data generated approximately 950,000 reads that passed quality control filters. Bacteroidia was the dominant class of bacteria amplified from the inland freshwater sample from the LA River (Fig. 2). This class was overtaken by Alpha- and Gammaproteobacteria at Site 2, where the mouth of the LA River meets the Pacific Ocean. Salinity levels characterized this site closer to saltwater. The Alpha- and Gammaproteobacteria class remained dominant for the rest of the sites. The SAR11 clade, the most abundant bacterial clades in the ocean[34–36], comprised 3.95% of total reads. Some other common indicator species (defined as observed > 10,000 times) from saltwater sites belonged to the genera Synechococcus, the SAR86 clade, and Rhodobacteraceae.

Alpha diversity analysis using the Shannon diversity index showed that the inland LA River sample had the lowest diversity of bacteria (H = 2.92) and Site 2, where the LA River meets the Pacific Ocean, had the highest diversity (H = 4.25, Fig. 3). Sites with increasing distance from Site 2 showed decreasing diversity, except for the furthest site (Site 8, H = 3.65). Beta diversity scored by the Jaccard dissimilarity index indicated high clustering of Sites 4-7, and Sites 1 and 2 were most dissimilar from the rest of the sites (Fig. 4). There were no sites that detected a species that was completely unique to a single site.

T-tests on MIC values revealed that marine bacteria had higher antibiotic resistance than those found in freshwater samples in 5 out of 12 antibiotic treatments: tetracycline, gentamicin, tobramycin, clindamycin, and cefoxitin (Fig. 5). Streptomycin and sulfamonomethoxine showed the opposite pattern where the freshwater sample had higher resistance than saltwater samples. Erythromycin was different in that the furthest marine site showed significantly higher resistance compared to all other sites. Ampicillin could not fit a dose response curve, meaning that very little susceptibility was observed in the concentrations initially tested. Experiments containing ampicillin were then rerun, with the highest concentration increased to 20,000 mg/mL. This was the highest antibiotic concentration that could be prepared without ampicillin precipitating out of solution. However, saltwater samples still showed minimal susceptibility at these high concentrations, so experiments using ampicillin were excluded from results. Pearson's correlation tests showed a positive correlation between MIC values and alpha diversity in 4 antibiotic treatments: gentamicin (r = 0.57), tobramycin (r = 0.64), clindamycin (r = 0.66), and levofloxacin (r = 0.51).

An ANOVA showed no significant differences of T_{opt} between all sites ($p = 0.38$), T_{min} ($p = 0.30$), or T_{max} ($p = 0.19$). Averages of T_{opt} at each site spanned 30.5-36.6 °C; T_{min} spanned 7.1-9.8 °C and T_{max} spanned 50.9-68.7 °C (Fig. 6).

Discussion

Our study found that bacteria from marine sites, further away from shore and anthropogenic sources of pollution, showed higher antibiotic resistance than bacteria from the LA River. This ran contrary to other studies that observed fewer antibiotic resistance gene abundances as distance from pollution sources increased[34,37]. A limitation of this observation is that running MIC experiments using bacteria grown in artificial media in the laboratory biased bacterial composition to represent only species that can be cultured. However, even if cultures may have filtered out species, we found it surprising to observe higher antibiotic resistance in bacteria that come from marine environments, which are more dissimilar to the laboratory environment. Some higher-end MIC values observed in marine sites were around 350 mg/mL in the gentamicin treatment group and nearly 3,000 mg/mL in the clindamycin treatment group. As a clinical reference, the Merck manual describes that usual IV dosages of gentamicin for adults as 5-7 mg/kg per day, or oral dosages of clindamycin as 150-450 mg every 6 hours[38].

Explanations behind observing higher resistance may be due to bacterial community composition or properties of the antibiotics used. Without whole genome sequencing, it is difficult to parse if certain species are responsible for higher resistance. However, we generally observed lower or equal resistance to 9 antibiotics in Site 1, which also showed the lowest alpha diversity. Higher diversity correlating with higher antibiotic resistance suggests high prevalence of antibiotic resistance genes shared across marine taxa. After observing high MIC values from

marine sites, we tested if saltwater bacteria may be thermally adapted, which is a trait known to also confer antibiotic resistance[32]. However, TPC experiments showed no evidence for thermal adaptation. Although evolving to environments with high salinity may also be adaptive for antibiotic resistance[37,39], measuring salinity as a gradient was not part of this study's experimental conditions.

Although marine bacterial communities display huge spatial and temporal variability, some common clades in marine bacterial composition can be observed. We observed bacteria that are typical in dominant clades in any given community: Alphaproteobacteria, Gammaproteobacteria, Actinobacteria, and Cyanobacteria[34,37,40]. While our study did not include a temporal element, studies on marine bacteria that include a large-scale time series are highly insightful. Marine bacterial communities exhibit high turnover at the seasonal scale as well as lower turnover at the interannual scale[35,41]. While coastal marine bacterial communities also show seasonality, they are also susceptible to stochastic events from anthropogenic influences and therefore are less predictable[42,43].

Some patterns in antibiotic drug classes and resistance that have emerged in our study echoed patterns observed in the clinical environment. High resistance in marine sites were seen in tetracycline and 2 out of 3 aminoglycosides, which share the same mechanism of action. In the clinical environment, first- and second-generation tetracyclines have lost efficacy due to the growing prevalence of ribosomal protection mechanisms[44]. High resistance in marine sites was also seen in cefoxitin as well as ampicillin, which showed such little susceptibility that an MIC value could not be generated. Both of these antibiotics are beta-lactams, and bacteria that are resistant to this antibiotic class are called extended-spectrum beta lactamase (ESBL) producers. These types of bacteria are under high surveillance because of the growing prevalence of *E. coli*

being ESBL producers[45]. These patterns also reflect frequent cross-resistance between tetracyclines and beta-lactams[44].

Conclusion

Our study is one of few studies assessing antibiotic resistance as a phenotype of whole bacterial communities sampled from marine sites. As we did not find a relationship between distance from shore and the strength of antibiotic resistance, we highlight how prevalence of antibiotic resistance does not always follow a distance dilution and that transmission of antibiotic resistance is due to a multitude of complex factors. As we were surprised by our findings, we speculate if the strength of antibiotic resistance that we have observed in marine bacteria could be due to an environmental variable we have overlooked, or if it could possibly be indicative of gene transfer agents that facilitate faster horizontal gene transfer[19]. There lies huge potential in the ocean to understand the role the environment itself plays in the spread of antibiotic resistant bacteria.

We also highlight the need for further understanding of the mechanisms and genetics behind antibiotic resistance in marine bacterial communities. While we have data on community composition and resistance phenotypes, a way to bridge the two would be through measuring antibiotic resistance gene quantity using qPCR[46]. This method could probe for genes that are resistant for the same antibiotics used in the MIC experiments, as well as genes involved in horizontal gene transfer that is often used as an urban marker[47,48]. As a next step, qPCR could provide a means to correlate the genotype of antibiotic resistance in a bacterial community with its phenotype, as well as a way to further describe differences in antibiotic resistance between freshwater and saltwater environments.

Figures and Tables

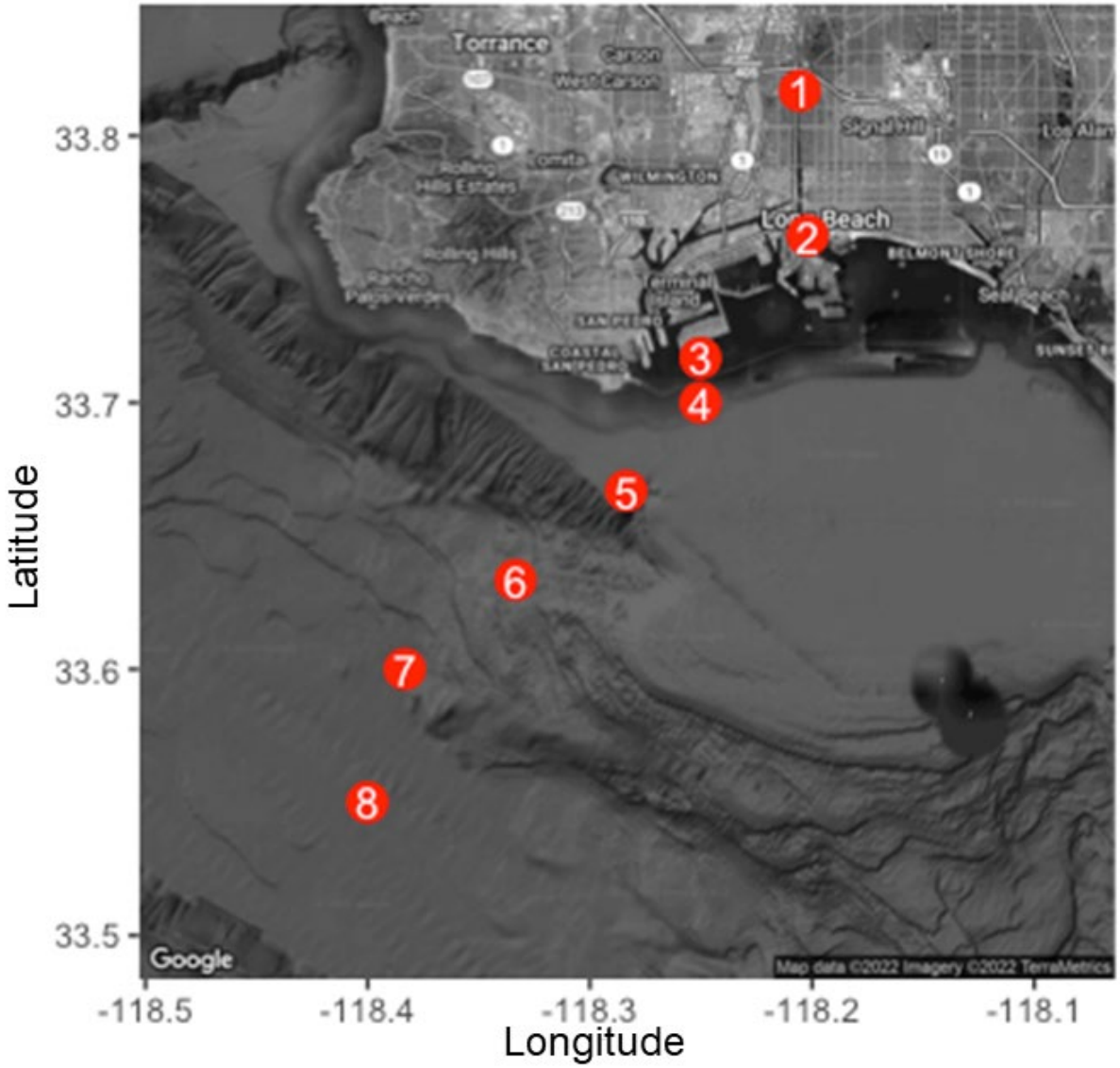

Figure 1. Water sampling sites in the LA River and Pacific Ocean. Site 1 in the LA River was designated as a freshwater sample and Sites 2-8 were designated as saltwater samples for experimental conditions and statistical analyses.

Table 1. Characteristics of water collected from each site.

Site	Distance from Site 2 (km)	Temperature (ºC)	Salinity (mS/cm)	pH
1	-6.049	30	0	10.43
2	0	26.8	31	7.74
3	6.775	24.4	35	8.13
4	8.264	24.7	35.5	8.22
5	13.041	24.3	35	8.19
6	18.815	24	36	8.17
7	24.665	24.5	35.5	8.17
8	29.915	24.2	36	8.19

Table 2. List of antibiotics used for MIC experiments.

Antibiotic	Class	Mechanism
Tetracycline	Tetracycline	Inhibition of protein synthesis by targeting 30S subunit
Gentamycin	Aminoglycoside	
Streptomycin	Aminoglycoside	
Tobramycin	Aminoglycoside	
Clindamycin	Lincosamide	Inhibition of protein synthesis by targeting 50S subunit
Erythromycin	Macrolide	
Ampicillin	Beta-lactam	Inhibition of cell wall synthesis
Cefoxitin	Beta-lactam	
Ciprofloxacin	Fluoroquinolone	Disruption of DNA gyrase function
Levofloxacin	Fluoroquinolone	
Sulfamonomethoxine	Sulfanomide	Inhibition of folic acid synthesis
Trimethoprim	Diaminopyrimidine	Inhibition of folic acid metabolism

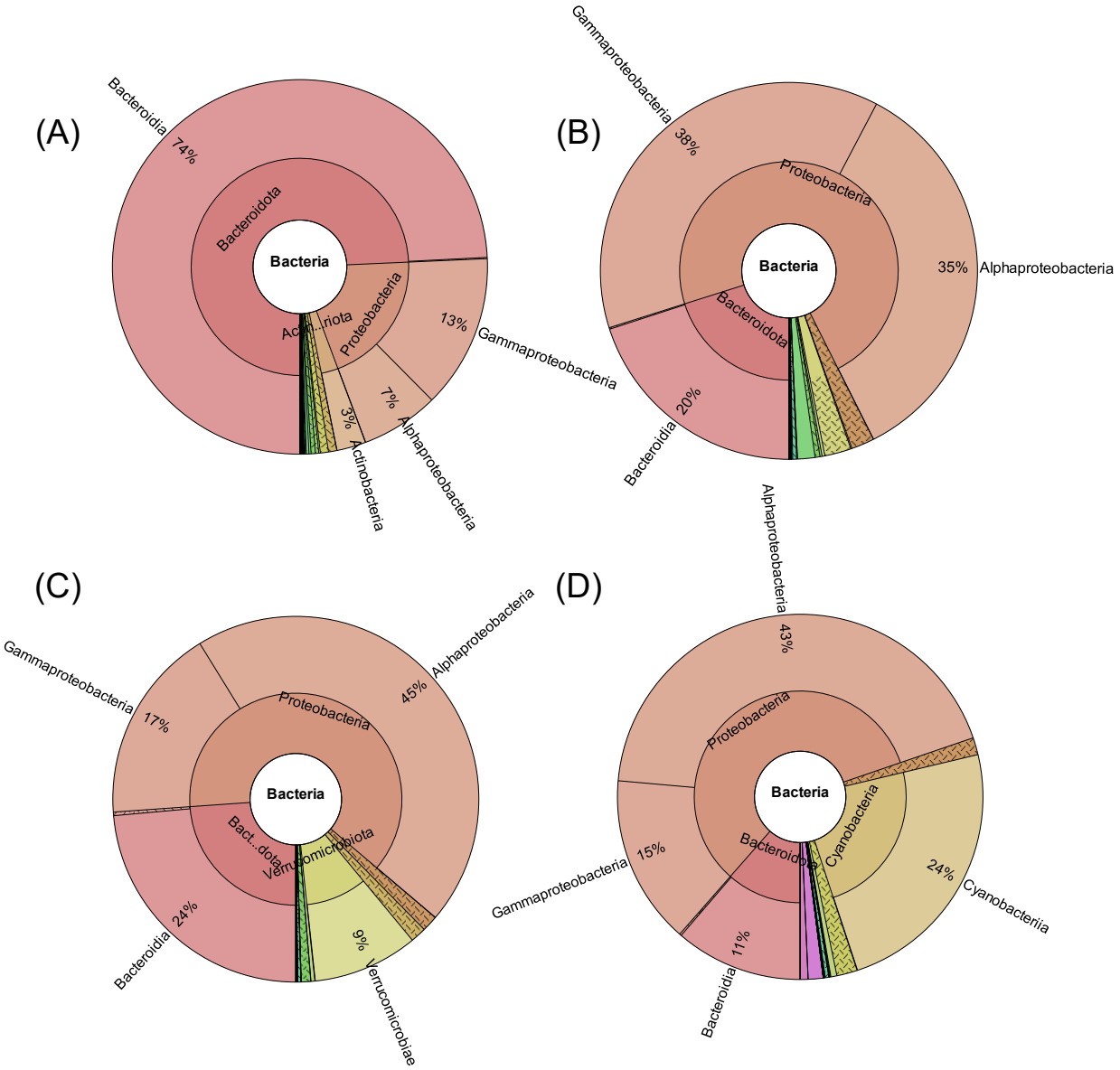

Figure 2. Relative counts of bacteria at the class level in samples from four sites. (A) Site 1 (LA River). (B) Site 2 (mouth of the LA River). (C) Site 3 (marine site closest to shore). (D) Site 8 (furthest marine site from shore, in the middle of the San Pedro Channel). As samples changed from freshwater to saltwater, dominance of the Bacteroidia clade was overtaken by the Proteobacteria clade.

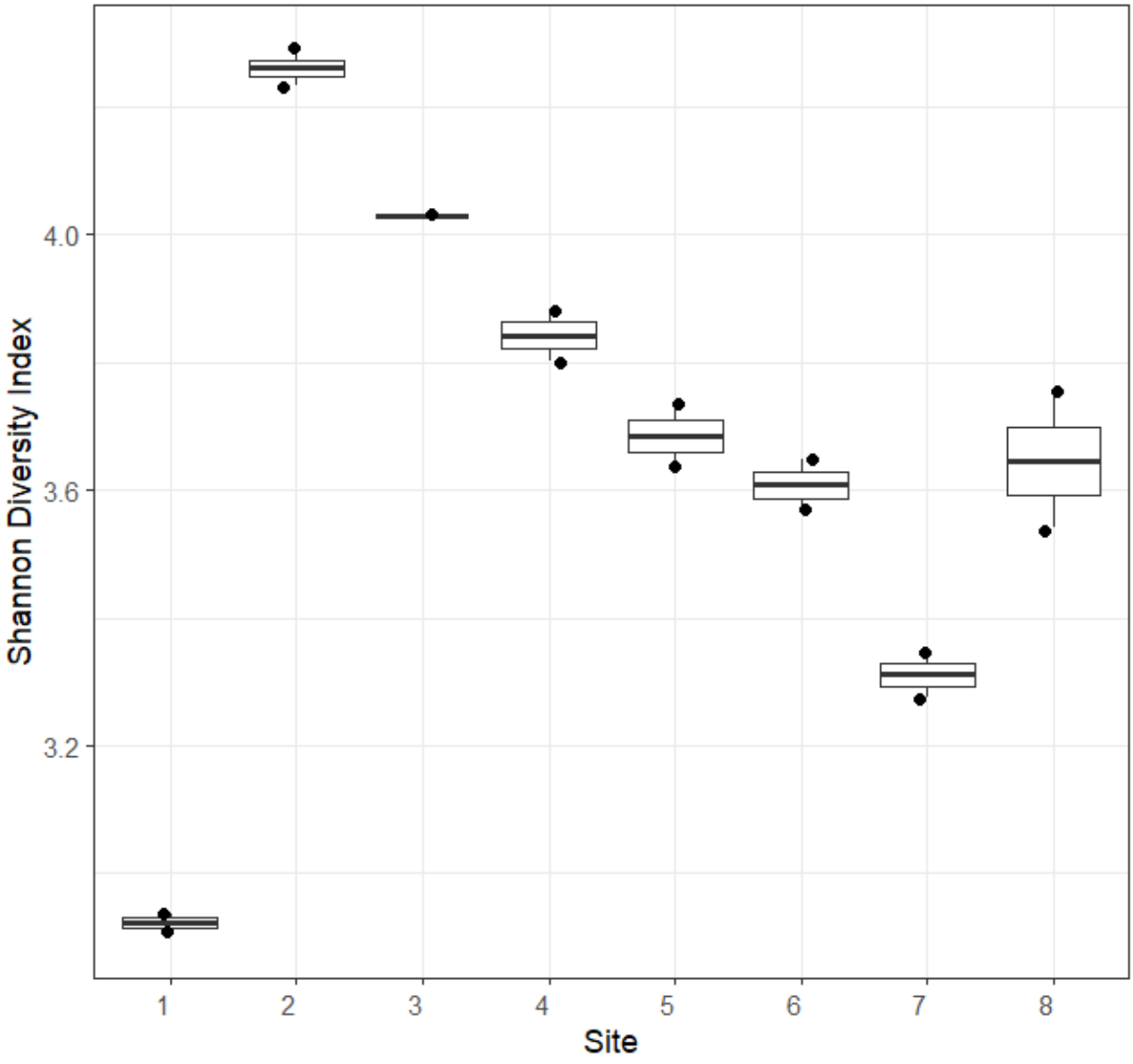

Figure 3. Shannon Diversity Index of alpha diversity at each sampling site. Site 1 collected from the LA River was appreciably less diverse than all other sites.

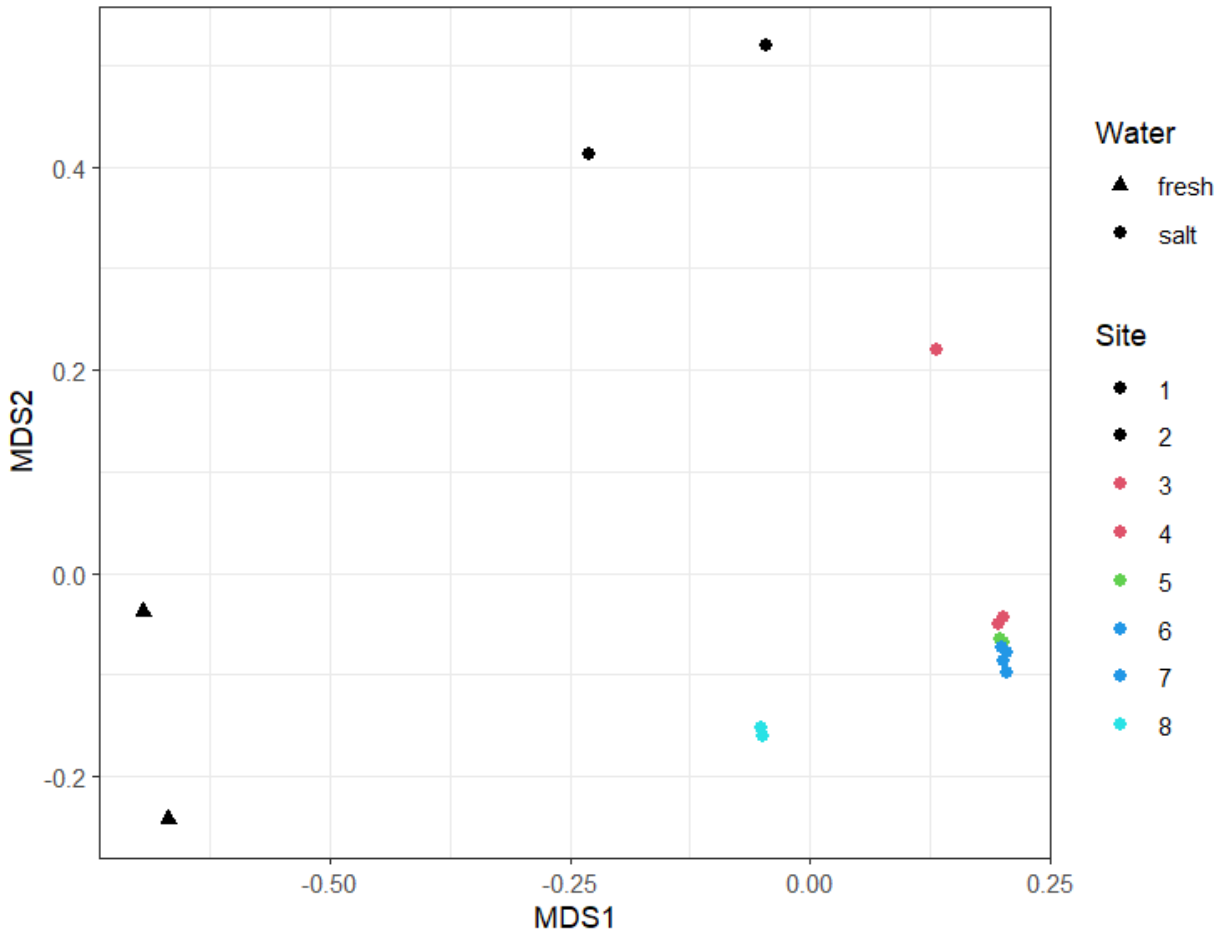

Figure 4. Jaccard dissimilarity index measuring beta diversity between sampling sites. Grouping of diversity could be seen in Sites 4-7. Sites 1 and 2 were most dissimilar from each other and the rest of the sites. Hypothesis testing for beta diversity using PERMANOVA rejects the null hypothesis that the centroids and dispersions of MDS between all sampling sites are equivalent ($p = 0.01$).

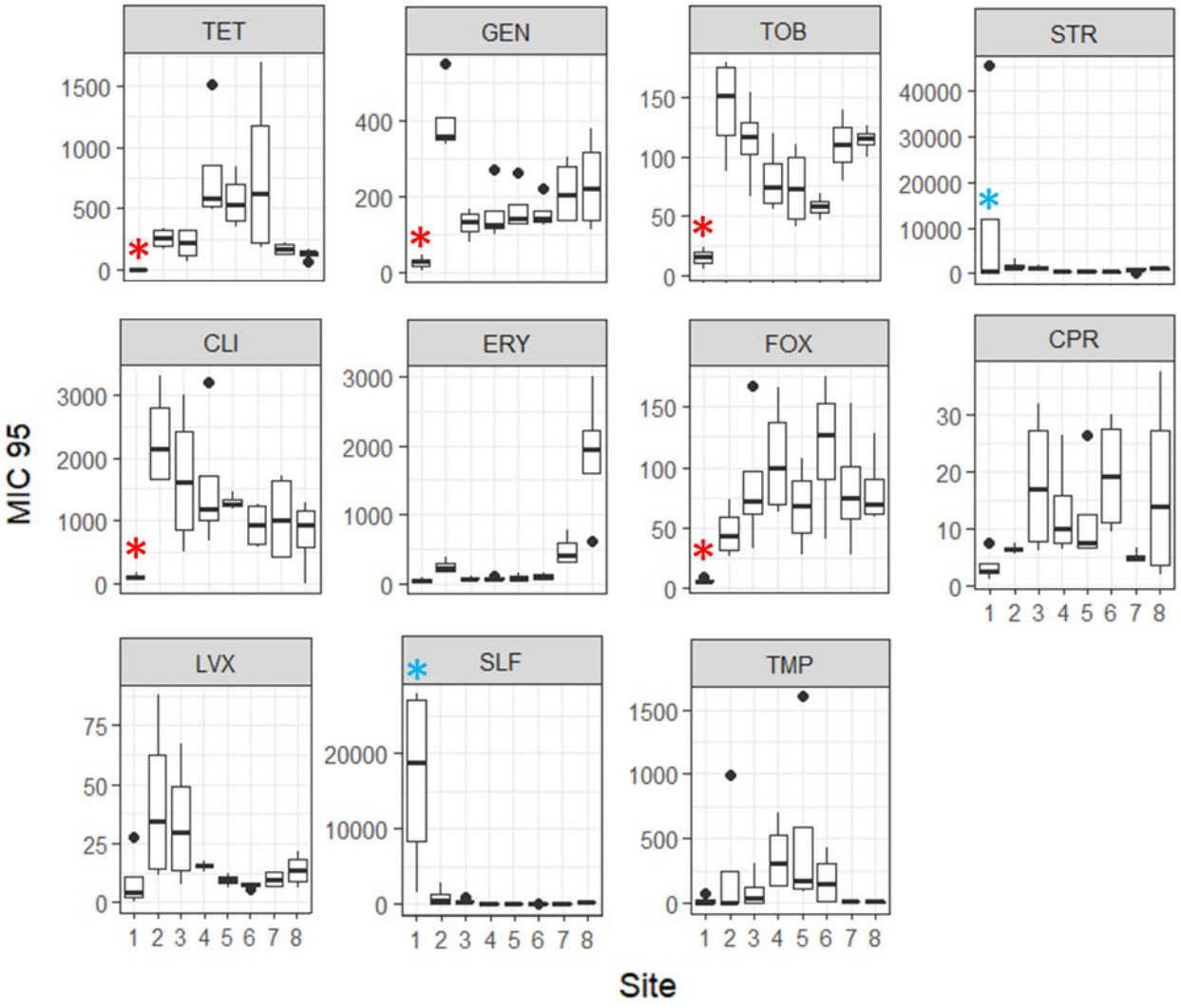

Figure 5. MIC values showed higher resistance in marine sites compared to freshwater sites in 5 out of 11 antibiotic treatments. Red asterisks denote that Site 1 MIC values is significantly different lower from the rest of the sites. Blue asterisks denote that Site 1 MIC values is significantly higher from the rest of the sites. Site 8, the furthest marine site, showed significant difference in resistance for erythromycin. Antibiotic names are abbreviated as TET = tetracycline, GEN = gentamicin, TOB = tobramycin, STR = streptomycin, CLI = clindamycin, ERY = erythromycin, FOX = cefoxitin, CPR = ciprofloxacin, LVX = levofloxacin, SLF = sulfamonomethoxine, TMP = trimethoprim.

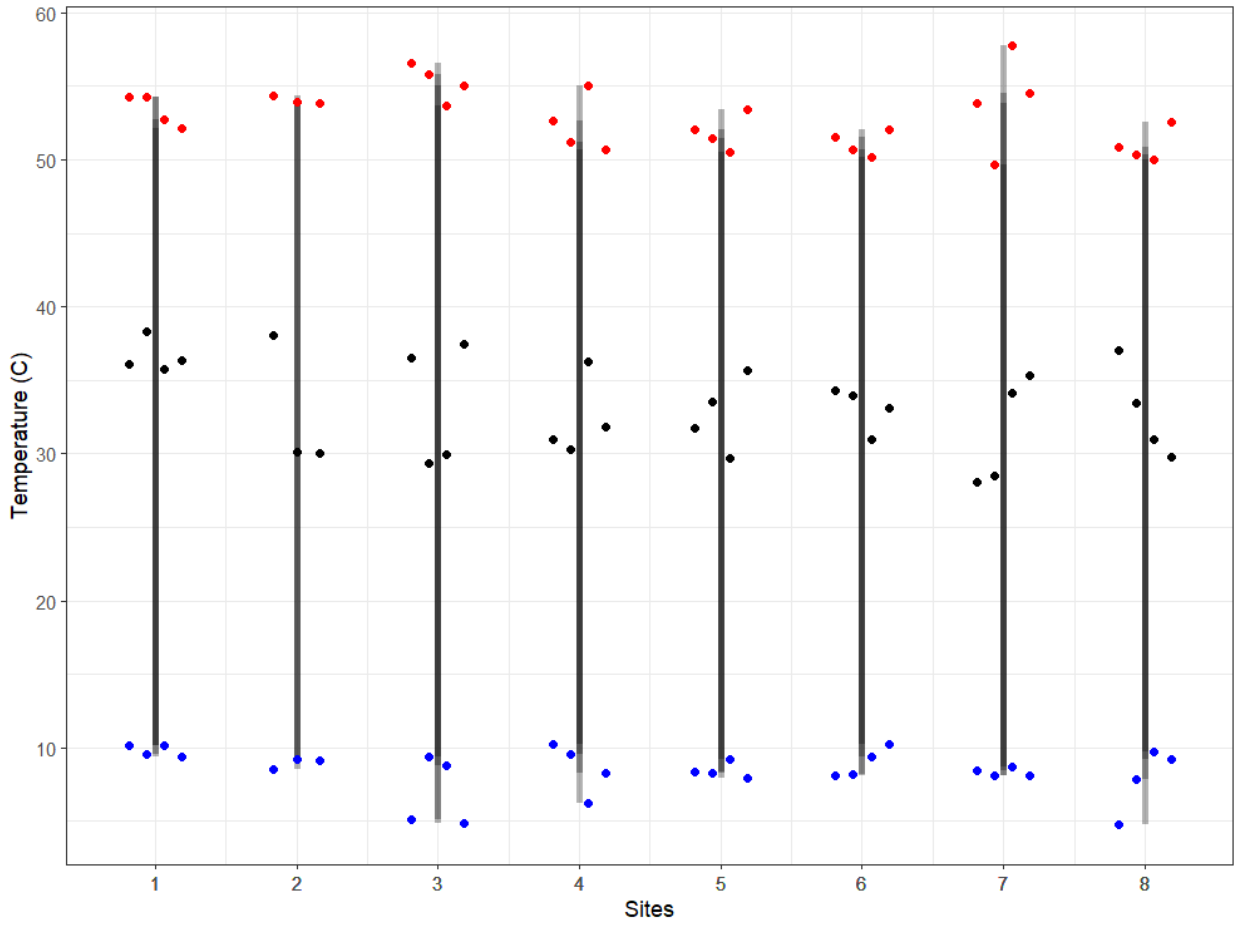

Figure 6. T_{opt} (black), T_{min} (blue), and T_{max} (red) were not significantly different between all sites after a 24 h time series experiment. This lack of difference indicates that marine bacteria were not more thermally adapted than freshwater bacteria, or vice versa.

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