

Article

Metagenomics Reveal Dynamic Coastal Ocean Reservoir of Antibiotic Resistance Genes

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Abstract: Exposure to antibiotic-resistant microbial communities in coastal waters is an important threat to human health. Through a ten-year coastal time series, we used metagenomics from 236 time points to provide a comprehensive understanding of the seawater resistome, temporal distribution, and factors influencing frequencies of specific resistance types. Here, we predicted that antibiotic resistance gene frequencies would increase during the winter due to increased rainfall, with terrestrial and enteric taxa serving as the primary carriers of resistance genes in coastal waters. We found that seasonal and interannual trends of antibiotic resistance genes vary by gene and the taxa carrying them, as opposed to a general increase in most resistance genes during specific seasons. However, we found that precipitation and *Enterococcus* levels may be accurate indicators for total antibiotic resistance gene levels in Newport Beach coastal water. Resistance genes were primarily carried by marine taxa, though some terrestrial taxa and opportunistic pathogens also harbored these genes. Non-marine taxa can be introduced through rain, human activity, or sewage spills. By using metagenomics, we were able to elucidate the antibiotic-resistant bacterial communities in Newport Beach coastal water and demonstrate both seasonal and multiannual trends in their abundance with important implications for local health and safety.



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1. Introduction

Through runoff of pollutants, such as treated or raw sewage, coastal waters may pose an intermittent or continuous risk to human health through exposure to antibiotic-resistant bacteria. The influx of antibiotics and antibiotic-resistant bacteria into the ocean forms the basis for a reservoir of resistance genes that may be incorporated into marine microbial genomes via horizontal gene transfer [1]. During recreational activities at the beach, people may ingest bacteria harboring antibiotic resistance (AR) genes, which in turn can lead to infection and/or transfer of genes to commensal and opportunistic pathogens resident in the human microbiome [1,2]. Additionally, climate change can increase the frequency of AR genes [3–5]. Climate change is driving an increase in high-precipitation events. Such events increase flooding and stormwater runoff, transporting bacteria and substances that may promote the development of antibiotic resistance in seawater [3]. An increase in temperature was shown to increase antibiotic resistance among common pathogens [4,5] and can lead to antibiotic resistance in the lab, even in the absence of

antibiotics [6]. However, few studies have thoroughly studied the prevalence of AR genes in coastal water at a single location over an extended period of time and, thus, the seasonal and multiannual trends in coastal AR gene dynamics are largely unknown.

Seawater has been shown to be a reservoir of AR genes [7–22]. Specifically, coral reef regions and deep and coastal ocean water have been shown to harbor AR genes. Significantly higher abundances of AR genes have been found in coastal water compared to deep ocean and Antarctic seawater samples, highlighting the pressure exerted from human activity on the biogeography of AR genes [7]. However, antibiotic-resistant and virulent *Staphylococcus aureus* have been found in Hawaiian beaches with limited human activity [22]. Many beta-lactamases, including those uncommonly found outside of clinical environments, have been found at Australian beaches [18]. Multi-resistant *Escherichia coli* were found at a touristic island in Portugal, and this reservoir of AR genes was mainly modulated by seagull-derived fecal pollution [13]. Among Southern Californian beaches, about 45% of total coliforms isolated were resistant to ampicillin and remained consistent during dry and wet seasons [10]. Through functional metagenomics, Newport Beach, California was shown to harbor a diversity of known and unclassified AR genes carried by marine and non-marine taxa [15]. However, the majority of these studies analyzed samples collected within only one year or with culture-based techniques [10,11,13–15,17,22]. Metagenomics is an alternative but powerful tool for assessing AR genes as it captures the diversity of all resistance mechanisms and the taxa carrying these genes [7,8,16,23] and is not restricted to studying culturable bacteria with limited antibiotic resistance mechanisms. Metagenomics is also less labor intensive and can generate large volumes of microbial data relative quickly [16]. However, it should be noted that, although data generation for metagenomics is straightforward, quantifying low variance and sample bias for metagenomic data can be a challenge, and that quantitative standardization techniques are relatively new and can be difficult to implement. An examination of AR genes via metagenomics over multiannual seasonal cycles may help reveal the periodicity and prevalence of potential health risks posed by AR genes in coastal waters.

Newport Beach, CA is an ideal location to study coastal AR genes because it is surrounded by one of the most densely populated communities and largest harbors for recreational vessels in the United States [24]. This study location is part of the broader California Current Ecosystem, a mid-latitude eastern boundary upwelling system. More specifically, the study location is in the Southern California Bight, which is largely impacted by the southward, longshore, cold, and nutrient-depleted California Current, but also by the northward, warm, and nutrient-enriched California Undercurrent [25]. Previous studies conducted at the “Microbes in the Coastal Region of Orange County” (MICRO) time-series station at Newport Pier, California have demonstrated how seasonal variability and multi-annual ocean warming driven by El Niño and climatically forced temperature anomalies lead to shifts in nutrient availability, organic matter composition and concentrations, and microbial community composition [25–28]. We hypothesize that seasonal changes will greatly impact AR genes in coastal water. For example, we predict that antibiotic resistance frequencies will increase during rain events in the winter and during high-beach-usage months in the summer. We also predict that terrestrial and enteric taxa that wash in during rain events or carried by visitors during the summer are the main sources of AR genes in coastal water.

Here, we used a ten-year time series to determine the prevalence of AR genes in Newport Beach, CA coastal water using metagenomics. We specifically asked the following: (i) What are seasonal changes and associated impact of the number of visitors present on the beach or *Enterococcus* levels on the frequency of AR genes? (ii) Which taxa harbor AR genes? We seek to delineate the taxa of origin for AR genes that are present in coastal water

and their temporal modes of variation, as well as pinpoint when and why their frequencies increase. By characterizing temporal trends in AR genes, we hope to provide resource managers and others with greater information on the potential hazards posed by antibiotic resistance in coastal waters.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

Between 2011 and 2020, 236 surface seawater samples were collected weekly to monthly from the “Microbes in the Coastal Region of Orange County” (MICRO) time-series station at Newport Pier in Newport Beach, California, USA (33.608°N and 117.928°W). Samples were collected and processed as previously described [25–27]. Bacterial DNA was extracted from Sterivex syringe filters (Millipore, Burlington, MA, USA) as described previously [25]. Briefly, the filters were incubated at 37 °C for 30 min with lysozyme (50 mg/mL final concentration) prior to adding Proteinase K (1 mg/mL) and 10% SDS buffer, then incubated at 55 °C overnight. DNA was precipitated using ice-cold isopropanol (100%) and sodium acetate (245 mg/mL, pH 5.2). DNA was then centrifuged and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA) in a 37 °C water bath for 30 min. DNA was purified using a genomic DNA Clean and Concentrator kit (Zymo Research Corp., Irvine, CA, USA) and then quantified using a Qubit fluorometer (ThermoFisher, Waltham, MA, USA). DNA quality (30–60 ng) was assessed via visual examination using gel electrophoresis as well as a Nanodrop ND-1000 (ThermoFisher, Waltham, MA, USA). All products submitted for sequencing had A260/A280 values between 1.6 and 2.0 and A260/A230 values between 2.0 and 2.2. Frozen DNA in 1xTE suspension buffer was overnighted on dry ice to the Joint Genome Institute for Sequencing.

2.2. Library Preparation, Sequencing, and Analysis

The U.S. Department of Energy Joint Genome Institute (JGI) performed the library preparation, sequencing of metagenomes, processing of reads, and functional and taxonomic annotations according to their metagenome workflow [29]. For detailed protocols, see Larkin et al. [28]. In brief, sequencing was performed on the Illumina NovaSeq 6000 S4 flowcell using NovaSeq XP V1.5 reagent kits and 2 × 151 indexed chemistry. A sequencing depth of 13.6 Gbp/sample was achieved for a total of 3.21 Tbp. Sequence data is available through the JGI Genome Portal (<https://www.osti.gov/award-doi-service/biblio/10.46936/10.25585/60001365>, accessed on 3 January 2023).

Raw reads were processed using BBDuk version 38.94 from the BBTools package (<https://jgi.doe.gov/data-and-tools/software-tools/bbtools/>, accessed on 3 January 2023) to remove adapters, perform quality trimming, and remove reads with >4 “N” bases, an average quality score < 3, a minimum length < 51, and homopolymer stretches of 5 Gs or more. Filtered reads were assembled with metaSPAdes version 3.15.0. Contigs with a length < 200 bp were removed. An average of 71.99% quality-filtered reads (Minimum: 22.67%, Maximum: 100%) was assembled. To obtain coverage information, assembled reads were mapped to the contigs using ‘bbmap’ from BBTools (v38.94). Assembled contigs were then passed onto the annotation module of the workflow, which first predicts noncoding RNA genes, followed by the identification of clustered regularly interspaced short palindromic repeats (CRISPR) and protein-coding genes (CDSs). Structural annotation was predicted using Prodigal 2.6.3 [30], GeneMarkS-2 1.07 [31], tRNAscan-SE (v2.0) [32], RFAM [33], and CRT-CLI (v1.8). The last step of the feature prediction module combines the results from all tools and attempts to resolve overlaps between features of different types to produce a consensus structural annotation.

Functional annotation for metagenomes was performed by associating protein-coding genes with KO (KEGG Orthology) terms [34], Enzyme Commission (EC) numbers [35], COG (Cluster of Orthologous Genes) assignments [36], TIGRFAM [37], and Pfam [38]. Genes are associated with functional annotation terms based on the results of a sequence similarity search of metagenome proteins against a reference database of isolate proteomes using the large-scale alignment tool, LAST [39]. The best LAST hits of CDSs were also used for the taxonomic annotation of metagenomes. The taxonomy of best hit was assigned to each metagenome protein. JGI's pipeline can be implemented using the National Microbiome Data Collaborative's (NMDC) open-source online platform Empowering the Development of Genomics Expertise (EDGE) [40].

2.3. Antibiotic Resistance Analysis

We obtained a total of 102 AR KOs from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [41–43]. These AR KOs included beta-lactamase, aminoglycoside, multidrug, trimethoprim, macrolide, and tetracycline resistance mechanisms (Table S1). We found that 78 of 102 selected KOs were present within less than 5% of samples or were completely absent and removed from further analysis. The remaining 24 KOs were present in at least 5% of samples. We referred to AR KOs by the gene(s) to which they correspond (Table 1). We determined the frequencies of these 24 AR genes using their respective raw coverage values (Figure 1). Coverage was defined as the average coverage of the gene within the contig. Raw gene coverage values were normalized by the total sequence depth for each sample. Normalized coverage was scaled zero to one to conduct regression analysis.

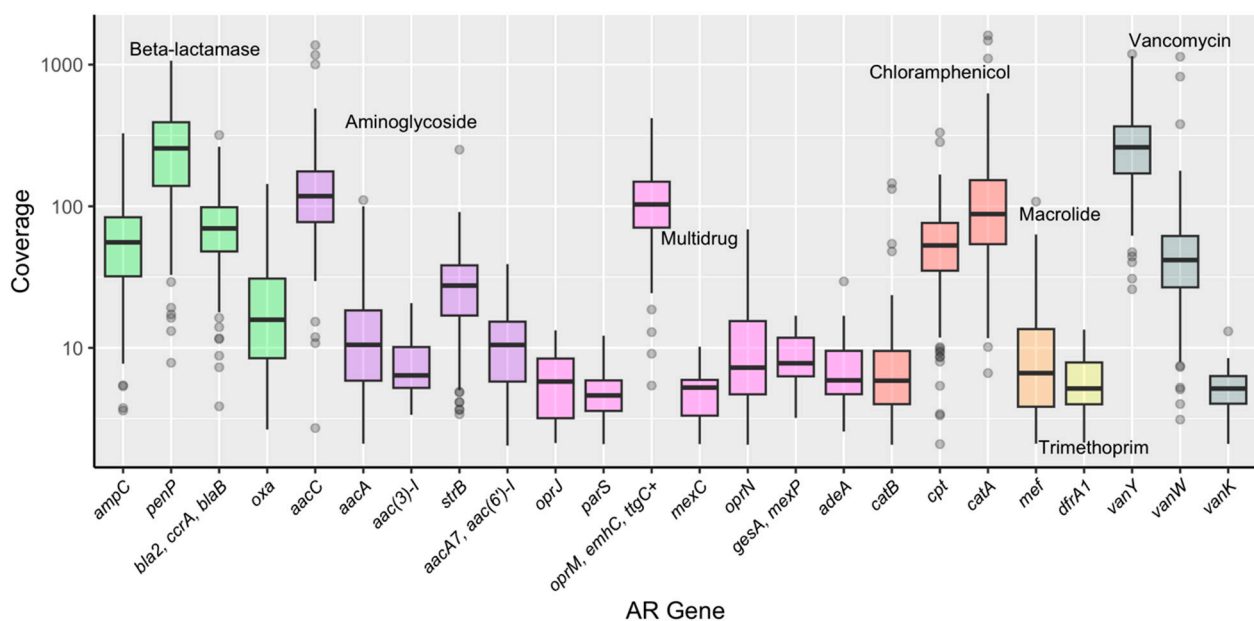


Figure 1. Raw coverage of antibiotic resistance genes present in at least 5% of samples. Coverage is defined as the average coverage within the contig that contains the gene. For example, a coverage of one is an average of one read per base pair in the contig. Genes are color-coded by resistance mechanism. The boundary of the box closest to zero indicates the 25th percentile, and the boundary of the box farthest from zero indicates the 75th percentile. The black line within the box marks the median. Points indicate outliers.

Table 1. Antibiotic resistance genes and the taxa harboring them. The environment (Env.) columns indicates the environment(s) from which the taxa has been isolated: A = Aquatic, E = Environmental, non-aquatic, H = Host-associated.

ARG Type	KO ^a	Gene ^a	Genus 1 ^b	Env. ^b	Genus 2 ^c	Env. ^c
Beta-lactamase	K01467	<i>ampC</i>	<i>Pseudohongiella</i>	A	<i>Thalassomonas</i>	H
	K17836	<i>penP</i>	<i>Synechococcus</i>	A	<i>Chryseolinea</i>	E
	K17837	<i>bla2, ccrA, blaB</i>	<i>Woeseia</i>	A	<i>Pseudohongiella</i>	A
	K17838	<i>oxa</i>	<i>Pseudohongiella</i>	A	Unclassified <i>Verrucomicrobiae</i>	A/E/H
Aminoglycoside	K00662	<i>aacC</i>	<i>Campylobacter</i>	A	<i>Lishizhenia</i>	A
	K00663	<i>aacA</i>	<i>Cognatijoonia</i>	A/E	<i>Candidatus</i> <i>Planktophila</i>	A
	K03395	<i>aac(3)-I</i>	<i>Sphingopyxis</i>	E	<i>Alcanivorax</i>	A
	K04343	<i>strB</i>	<i>Devosia</i>	E	<i>Drancourtella</i>	H
	K18816	<i>aacA7, aac(6')-I</i>	<i>Rhizobium</i>	E	<i>Salmonella</i>	H
Multidrug	K08721	<i>oprJ, oprM, emhC, ttgC, cusC, adeK, smeF, mtrE, cmeC, gesC</i>	<i>Pseudomonas</i>	A/E/H	<i>Marinobacter</i>	A
	K18139	<i>mexC</i>	<i>Pseudomonas</i>	A/E/H	<i>Psychrobacter</i>	A/E/H
	K18295	<i>parS</i>	<i>Pseudomonas</i>	A/E/H	<i>Halioglobus</i>	A/H
	K18072	<i>oprN</i>	<i>Pseudomonas</i>	A/E/H	<i>Bradyrhizobium</i>	E/H
	K18300	<i>gesA, mexP</i>	<i>Salinisphaera</i>	A	<i>Pseudomonas</i>	A/E/H
	K19595	<i>adeA</i>	Unclassified <i>Haliaceae</i>	A	<i>Limibacillus</i>	E/H
	K18145	<i>catB</i>	SAR116 <i>Halomonas</i>	A	<i>Halomonas</i>	A
	K00638	<i>cpt</i>	<i>Henriciella</i>	A	<i>Acinetobacter</i>	A/E/H
Chloramphenicol	K18554	<i>catA</i>	<i>Formosa</i>	A	<i>Streptomyces</i>	A/E/H
	K19271	<i>mef</i>	<i>Gordonibacter</i>	A	<i>Mucilaginibacter</i>	A/E
Macrolide	K08217	<i>dfrA1</i>	<i>Vibrio</i>	H	<i>Rhodoluna</i>	A
Trimethoprim	K18589	<i>vanY</i>	<i>Synechococcus</i>	A	<i>Prochlorococcus</i>	A
Vancomycin	K07260	<i>vanW</i>	<i>Flavobacterium</i>	A	<i>Synechococcus</i>	A
	K18346	<i>vanK</i>	<i>Catenulispora</i>	E	<i>Nocardioides</i>	A/E/H
	K18354					

^a Antibiotic resistance KOs and their respective gene names were obtained from KEGG. ^b The taxa hosting the highest percentage of coverage for the corresponding gene. ^c The taxa hosting the second highest percentage of coverage for the corresponding gene.

To quantify both seasonal (monthly reoccurrences across years) and interannual trends in the AR genes, we fit the following ANOVA model to our data [25]:

$$Y_{ijk} = \mu + \alpha_{\text{Year}}X_{\text{Year},j} + \beta_{\text{Month}}X_{\text{Month},k} + \varepsilon_{ijk}$$

We conducted Type II ANOVAs on categorical linear regressions of AR genes (Y_{ijk}) as a function of the year (X_j) and month (X_k) with corresponding regression coefficients α_{Year} and β_{Month} . R's "car" and "stats" packages were used to perform this analysis. Linear model regression coefficients were scaled to center on zero prior to plotting Figure 2.

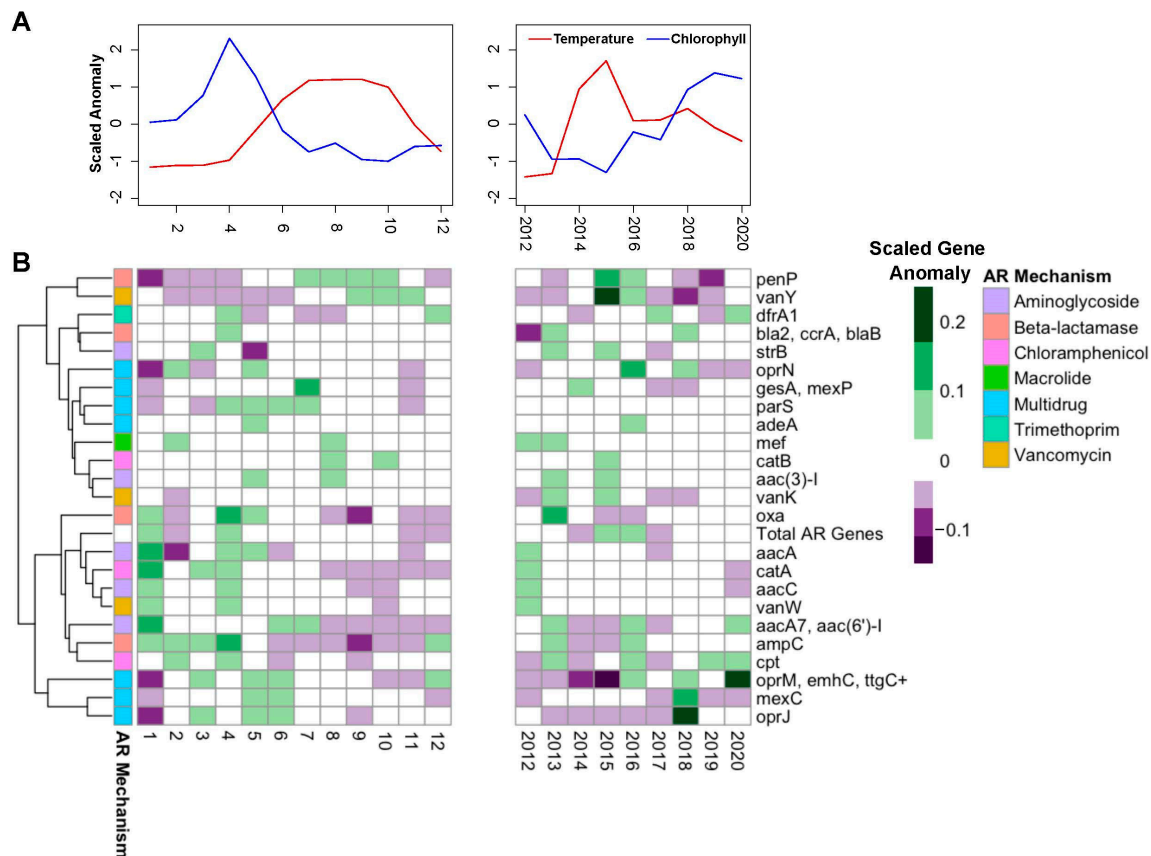


Figure 2. (A): Monthly and annual anomaly in temperature (red line) and chlorophyll (blue line). (B): Seasonal and interannual trends in antibiotic resistance genes present in at least 5% of samples. Linear model regression coefficients for normalized gene coverage as a function of month (1–12) and year (2012–2020) (see Methods). Each month is aggregated across years. Coefficients were scaled to center on zero. The regression model uses January and the first year of the time series (2011) as the reference category for month and year coefficients, respectively. A higher coefficient means an increasing trend in that month or year. Genes are color-coded by resistance mechanism. Anomalies were found by fitting linear models with 12 monthly and yearly levels, centered, and scaled for presentation.

2.4. Environmental Analysis

Precipitation records at Newport Pier in Newport Beach, CA from 2011 to 2020 were obtained from the National Oceanic and Atmospheric Administration. Temperature and chlorophyll were recorded via the Southern California Coastal Observing Systems (SC-COOS) automated shore station (Newport Pier) located on Newport Pier. Data documenting the number of visitors at Newport Beach, CA from 2015 to 2020 were obtained as a public records request from the City of Newport Beach. The recorded levels of *Enterococcus* at the 15S station located at 15th/16th St. in Newport Beach, CA from 2011 to 2020 were obtained from the State of California Water Boards.

The correlations between AR gene coverage and *Enterococcus* levels, number of people, and precipitation levels at Newport Beach, CA were quantified through the Pearson correlation coefficient. The linear model regression coefficients (unscaled) as a function of the month and year for AR gene coverage, *Enterococcus* levels, number of people, and precipitation levels were used to obtain the Pearson correlation coefficients.

3. Results and Discussion

Through a ten-year time series, we delineated the prevalence of AR genes in Newport Beach, CA coastal water ($n = 236$, Figure 1). We identified the seasonal (monthly reoccurrences across years) and interannual trends of AR genes (Figure 2) as well as the taxa harboring these genes (Table 1). We then examined the impact of rainfall, temperature, number of visitors on the beach, and *Enterococcus* levels (Figures 3 and 4) on the trends of AR genes. Therefore, we were able to characterize the presence of AR genes in coastal water and the variables potentially impacting them.

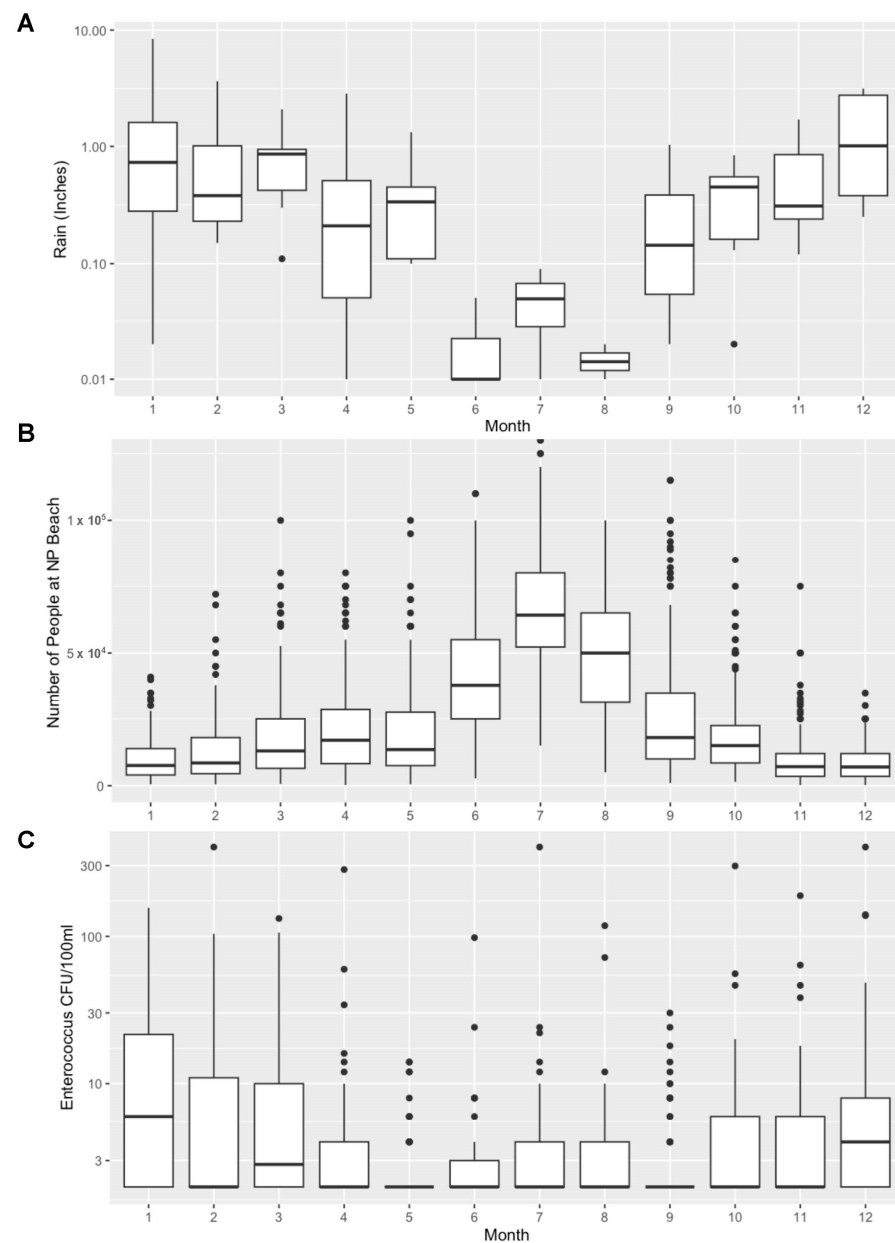


Figure 3. (A): Monthly precipitation at the Newport Beach Harbor, CA National Oceanic & Atmospheric Administration station from 2011 to 2020. Data for January 2011 and May 2012 is not available. (B): The number of monthly visitors at Newport Beach, CA from 2015 to 2020. Data was obtained as a public records request from the City of Newport Beach. (C): Monthly *Enterococcus* levels at the State of California Water Boards 15S station located at 15th/16th St. in Newport Beach, CA from 2011 to 2020. The boundary of the box closest to zero indicates the 25th percentile, and the boundary of the box farthest from zero indicates the 75th percentile. The black line within the box marks the median. Points indicate outliers.

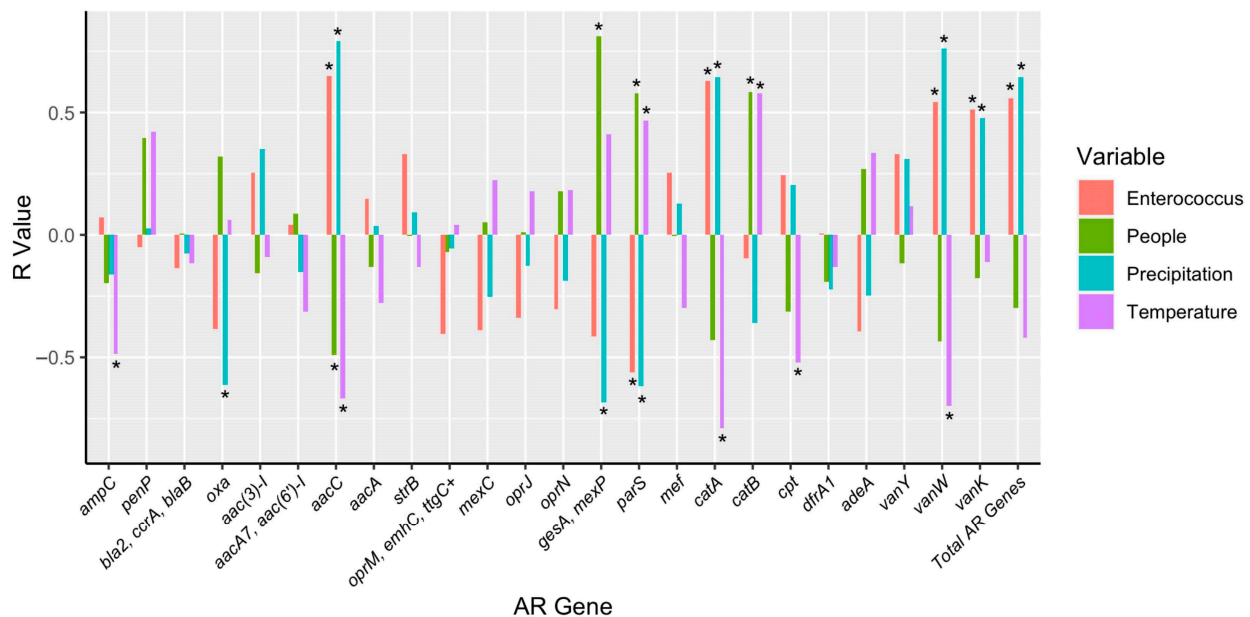


Figure 4. Pearson correlation coefficients (R values) for the correlation between AR gene coverage and *Enterococcus* levels, number of people, precipitation levels, and temperature at Newport Beach, CA. The linear model regression coefficients as a function of the month and year for AR gene coverage, *Enterococcus* levels, and precipitation levels were used to obtain the Pearson correlation coefficients. *, $p \leq 0.05$.

We detected a total of 24 out of 102 possible AR gene types that were present in at least 5% of samples. The 5% sample threshold is often applied to taxonomic filtering and has been shown to reduce the complexity of microbiome data while preserving downstream analysis, reduce technical variability, and result in more comparable and reproducible results [44]. One out of twenty-four AR genes did not consist of any genes present within the Comprehensive Antibiotic Resistance Database, which documents classified AR genes [45]. This gene is *penP* (Table 1), but *penP* is known to confer beta-lactam resistance in *Bacillus* [46]. We determined the coverage values of the 24 AR genes (Figure 1). Median coverage values ranged between 8 and 450 reads per gene bp. With an average sequencing depth of 13.6 Gbp per sample and average genome length of 2.36 Mbp per sample [28], the observed coverage indicates that between approximately 1:1000 and 1:13 cells contain at least one AR gene copy. The average prokaryotic cellular abundance in the euphotic zone at the nearby SPOT time series was 2×10^6 cells/mL over 10 years [47]. Thus, a very rough estimate of AR gene abundance in our time series is that it ranges between approximately 2.8×10^3 and 1.6×10^5 gene copies/mL. A recent transect from the western Pacific to the Southern Ocean used quantitative PCR to determine that AR gene abundances ranged between 1.4×10^6 and 7.8×10^6 copies/mL [48]. Therefore, our metagenomically estimated AR gene abundances were an order of magnitude lower and may have been impacted by detection limits, but were generally within the range expected for marine environments. We identified four beta-lactamase, five aminoglycoside, seven multidrug, three chloramphenicol, one macrolide, one trimethoprim, and three vancomycin resistance gene types. There was variability in coverage within each antibiotic category, but beta-lactamase and vancomycin resistance genes were the most prevalent (Figure 1). *penP* (class A beta-lactamase) and *vanY* (vancomycin resistance gene) had the highest coverage [41–43].

Specific groups of AR genes fluctuated in coverage seasonally and interannually (Figure 2). Genes presented a seasonality trend, remained constant, or displayed an inconsistent trend. For example, *oxa*, *aacA*, *catA*, *aacC*, *vanW*, *aacA7*, *aac(6')-I*, and *ampC*

displayed a potential seasonality as they had an increasing trend in January. Total AR genes also increased in January. *gesA* and *mexP* showed seasonality through a prominent increase in July. In addition, the two genes with the highest coverage, *penP* and *vanY* (Figure 1), demonstrated potential seasonality with their peak frequency in the late summer and fall. Genes that remained constant throughout the time series were *bla2*, *ccrA*, *blaB*, *adeA*, *mef*, *catB*, *aac(3)-I*, and *vanK*. Genes that displayed an inconsistent trend were *oprN*, *oprM*, *emhC*, *ttgC+* (K18139), and *oprJ*. The subsets of genes that displayed an increasing trend in May and June consisted primarily of multidrug resistance mechanisms, which may demonstrate a common driver of AR genes during these months. In 2015 and 2016, total AR genes increased. This may have occurred due to El Niño, which peaked in this period [25] and is characterized by an increase in sea surface temperatures. Thus, subsets of genes and AR mechanisms demonstrated trends in coverage levels linked to seasonal and/or interannual cycles.

Taxa harboring AR genes consisted predominantly of native/marine taxa, but there were also potentially pathogenic bacteria carrying AR genes (Table 1). Fourteen out of twenty-four AR genes were mostly represented within marine genera, which included *Pseudohongiella* [49], *Woeseia* [50], *Halomonas* [51], *Henriciella* [52], *Formosa* [53], *Vibrio* [54], *Synechococcus* [55], and *Flavobacterium* [56]. These genes have beta-lactamase, chloramphenicol, and trimethoprim resistance mechanisms. Three out of the five top genera carrying aminoglycoside resistance genes (*aac(3)-I*, *strB*, *aacA7*, and *aac(6')-I*) were primarily found in other non-host environments (excluding water sources). These genera are *Sphingopyxis* [57], *Devosia* [58], and *Rhizobium* [59]. *Sphingopyxis* and *Devosia* have been commonly found in oil and pesticide-contaminated soil. *strB*, *aacA7*, and *aac(6')-I* were also found within *Drancourtella* and *Salmonella*, respectively. This is noteworthy as *Drancourtella* and *Salmonella* reside within the intestines of humans and animals [60,61]. The top genus harboring *aacC* was *Campylobacter*. *Campylobacter* species are known pathogens in humans and animals [62]. However, only 5.6% of coverage pertaining to *aacC* was within *Campylobacter* (Table S2). Seventy-seven percent of coverage for *aacC* was found within reads not assigned to a genus (Table S2), and hence it is uncertain which taxon this gene mainly resided in. The top genus hosting macrolide resistance gene *mef* was *Gordonibacter* (43.8% of coverage). *Gordonibacter* resides in the human gut and has been isolated from the stools of patients who are healthy [63] or suffering from acute Crohn's disease [64]. Four of the seven multidrug resistance genes were found primarily (top genus) within *Pseudomonas* (Table 1). *Pseudomonas* species are commonly found in soil- and water-associated habitats and polluted environments, but they can also be opportunistic pathogens [65–68]. For example, *Pseudomonas* species such as *Pseudomonas putida* [67], *Pseudomonas pseudoalcaligenes* [65], and *Pseudomonas aeruginosa* [69] have caused infections in humans. Ninety-two percent of coverage for *oprJ* was found within *Pseudomonas* (Table S2). *OprJ* is the outer-membrane channel component of the MexCD-OprJ multidrug efflux complex. *P. aeruginosa* harbors this clinically relevant efflux complex, making it innately resistant to several antibiotics [70]. We found that the *Pseudomonas* species carrying these genes were primarily *P. pseudoalcaligenes*, *P. alcaliphila*, *P. putida*, and *P. balearica*. *P. pseudoalcaligenes* is a soil organism, but it is a rare opportunistic human pathogen [65]. *P. alcaliphila* typically resides in seawater [66] but has been isolated from patients with cystic fibrosis [71]. *P. putida* resides in soil- and water-associated environments, but it also rarely causes infection in humans [67]. *P. balearica* is usually found within aquatic and petroleum-polluted environments [68]. Thus, *Pseudomonas* carrying AR genes in Newport Beach coastal water are likely aquatic lineages but have the potential to cause infection in humans.

Our data suggest that seasonal and interannual trends of AR genes vary by gene and the taxa carrying them, but rainfall and *Enterococcus* levels may be an accurate indicator of

total AR gene levels. We hypothesized an increase in most resistance genes during specific winter and summer months. Stormwater events increased AR genes at multiple Australian beaches, and the corresponding genes were linked to pathogenic genera associated with wastewater [9,18]. Throughout the time series, Newport Beach had the highest rainfall during December and January (Figure 3). There was a significant positive correlation between precipitation at Newport Beach and *aacC*, *catA*, *vanW*, *vanK*, and total AR genes (Figure 4). Thus, precipitation is an indicator for total AR gene levels in Newport Beach coastal water. Newport Beach was visited more frequently during June, July, and August (Figure 3). The highest number of visitors occurred in July throughout the time series. We found a significant positive correlation between the number of people at Newport Beach and *gesA*, *mexP*, *parS*, and *catB* but not to total AR genes (Figure 4). Therefore, the number of visitors is not a good indicator of total AR gene levels in Newport Beach coastal water. In other global locations, the opposite trend has been identified. For example, a higher diversity of AR genes was found in an Antarctic freshwater zone with greater human activity compared to freshwater zones with little human intervention [72]. An increase in tourism at the Galapagos Islands led to increases in antibiotic-resistant *E. coli* and *Enterococcus* levels [73]. *Enterococci* are used as indicators of human fecal pollution in recreational waters as high concentrations of this genus are found in human feces [74], which has been linked to antibiotic resistance in aquatic environments [75]. Newport Beach may show differing trends, as *Enterococcus* concentrations were highest in the winter when tourism was at its lowest (Figure 3). Instead, high *Enterococcus* levels occurred when precipitation was highest at Newport Beach (Figure 3). Similarly, at an urbanized subtropical bay in Texas, rainfall was shown to be directly correlated with increased *Enterococcus* concentrations [75]. Even though *Enterococcus* levels increased with rainfall, we did not observe AR genes carried by *Enterococcus* throughout the time series (Table 1). Similarly, increased concentrations of *Enterococcus* were not correlated with an increase in antimicrobial-resistant *Enterococcus faecium* at an urbanized subtropical bay in Texas [75]. However, we found a significant positive correlation between *Enterococcus* levels at Newport Beach and *aacC*, *catA*, *vanW*, *vanK*, and total AR genes (Figure 4). Even though *Enterococcus* was not a carrier of AR genes, *Enterococcus* may reflect the dispersal of bacteria with AR genes and hence serve as an indicator of AR levels in Newport Beach coastal water.

Marine taxa are the primary sources of AR genes in Newport Beach coastal water. We had predicted that terrestrial and enteric taxa transported by people or terrestrial run-off, indicated by numbers of monthly beach visitors and rainfall, would be the primary carriers of AR genes. For example, colistin-resistant genes within Croatian coastal water were found to come predominantly from pathogenic genera [21]. In contrast, at Newport Beach we found that marine genera had the highest percentage of coverage for more than half of AR genes (Table 1). This study location is subject to dynamic tidal exchange of up to 6 m as well as currents exceeding 0.1 m s^{-1} , suggesting rapid water exchange between offshore and the coastal ocean [76,77]. Thus, we are likely observing microbial communities reflective of the wider region including the open ocean, which may contrast with sites that have longer coastal water residence times. Similarly, the majority of AR genes found within Los Angeles Harbor and the San Pedro Channel in CA came from marine taxa [15]. This previous study also demonstrated an equal distribution of AR genes coming from marine and nonmarine taxa in Newport Bay, CA coastal water [15]. We observed seasonally increasing trends of AR genes harbored within marine genera. For example, *Synechococcus* harboring *penP* showed elevated levels in July, August, September, and October (Figure 2). *Synechococcus* reproduces more quickly as temperature increases [78–80], complementing the increasing trend during the summer months, as observed in our study. *Synechococcus* hosting *penP* and *vanY* rose sharply in 2015 (Figure 2). This may have occurred due to El Niño, which

peaked in 2015 [25] and is characterized by an increase in sea surface temperatures. *Vibrio* carrying *dfrA1* increased in April and December (Figure 2). Conversely, *Vibrio* abundance is usually higher in the summer than in the winter [81]. *Pseudohongiella* harboring *oxa* and *ampC* displayed increasing trends from January to May and December (Figure 2). The relationship between seasonal taxonomic trends and AR gene dynamics may be reflected in the fact that temperature is only significantly correlated with some of the AR genes examined and not all AR genes (Figure 4). Therefore, we found that the temporal modes of variation for many AR genes in the coastal environment at Newport Beach may be driven by autochthonous marine taxa that grow optimally under specific conditions at both seasonal and multi-annual time scales.

A caveat of this study is that it is difficult to conclusively assign taxa to the genus level. There are large percentages of unassigned taxa hosting AR genes at the genus level (Table S2). However, it is common to have a large portion of unannotated reads from seawater samples as less than 10% of individual metagenomic reads from the ocean can be recruited to reference genomes [82]. Using metagenomics allows us to identify diverse AR genes as well as the taxa that carry them, but we cannot always fully identify their hosts [16,82]. Since we are using KO terms, a second caveat is that the antibiotic resistance function from the selected KOs is only a prediction. Some AR genes may carry other functions within the cell. Additionally, genes may require changes in regulation before conferring resistance.

4. Conclusions

Studies have shown that AR genes are a common occurrence in coastal water. Since Newport Beach in California is a highly visited beach, with up to 100,000 visitors each day in the summer (Figure 3), it is important to understand the prevalence of AR genes from a public health perspective. Metagenomics can facilitate the scanning of AR genes from diverse environments as well as become a tool for monitoring water quality [83]. Across more than 200 time points in a ten-year time series, we have provided a comprehensive understanding of the AR dynamics within Newport Beach seawater. This work suggests that rainfall and *Enterococcus* levels are the best predictors of total AR genes and are likely indicative of either the influx of allochthonous bacteria into the Newport Beach coastal water during the winter and spring through coastal runoff or an increase in autochthonous taxa carrying AR genes driven by favorable conditions (e.g., an increase in nutrients) (Figure 4). In contrast, in the late summer and fall as well as on multiannual timescales, marine taxa such as *Synechococcus* (Figure 2) drive the dominant signal in AR genes and appear to increase in abundance under warmer El Niño conditions. Given that median AR gene abundance was estimated to be on the lower end of marine observations and that the majority of AR genes were found in marine taxa, there is some evidence that management actions may not significantly reduce the prevalence of AR genes at this site. In contrast, the significant relationship between rainfall, *Enterococcus* levels, and total AR abundance suggests that improvements to wastewater management and treatment have the potential to reduce at least some of the AR gene prevalence at Newport Beach, thereby improving local health and safety. Overall, this study demonstrates that the temporal variability of AR genes is highly dependent on local environmental conditions and human dynamics, may vary from location to location, and may change over time due to both natural and anthropogenic environmental stressors.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/jmse13061165/s1>, Table S1: Total antibiotic resistance-related KOs classified by resistance mechanism; Table S2: Antibiotic resistance genes and the taxa harboring them.

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