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Quantifying Antibiotic Resistant Genes in Surface Coastal Waters

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Quantifying Antibiotic Resistant Genes in Surface Coastal Waters

A thesis in partial satisfaction

of the requirements for the degree Master of Science

in Civil and Environmental Engineering

by

Karina Jimenez

2021

ABSTRACT OF THE THESIS

Quantifying Antibiotic Resistant Genes in Surface Ocean Waters

by

Karina Jimenez

Master of Science in Civil and Environmental Engineering

University of California, Los Angeles, 2021

Professor Ertugrul Taciroglu, Chair

The presence of antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) in aquatic environments is cause for concern. Research studies have linked animal and human waste as a source for ARGs and ARB found in stormwater runoff, agricultural runoff, wastewater treatment plants, and hospital effluents. Several of these pathways connect to the ocean, potentially creating another reservoir for ARGs. Coastal environments are relied on for recreational uses, food, and more, but the relationship between pathogens present in coastal environments and human health have not been fully established. Thus, quantifying the amount of ARGs present in ocean water, particularly in surface coastal waters where humans are more likely to encounter any harmful pathogens, is pivotal to understanding and mitigating the threat

of antibiotic resistance in aquatic environments. This thesis is divided into two chapters. Chapter one is a systematic review for quantitative resistance data available for coastal surface waters. Chapter two investigates ARGs in ocean water at two California beaches.

The thesis of Karina Jimenez is approved.

Shaily Mahendra

Sanjay Mohanty

Jennifer Jay, Committee Chair

University of California, Los Angeles

2021

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Chapter 1. Quantifying Antibiotic Resistant Genes in Surface Ocean Water: A Systematic Review

Introduction

In the United States each year more than 2.8 million antibiotic-resistant infections occur, and more than 35,000 people die as a result (Centers for Disease Control and Prevention, 2019). Antibiotic resistance refers to the mutational changes in bacteria that render antibiotics ineffective in killing or stopping the growth of infections. Antibiotic resistant genes (ARGs) are the genetic material that encode defense mechanisms against certain antibiotics such as limiting drug uptake, modifying a drug target, inactivating a drug, or active drug efflux (Reygaert, 2018). ARGs can intrinsically be present in bacteria and can spread between bacteria through horizontal gene transfer. Extensive research has been done on ARGs and antibiotic resistant bacteria (ARB) to determine their fate and transport in different aquatic environments. There is evidence of a link between excessive consumption of antibiotics and higher levels of resistance (Kümmerer, 2009; Michael et al., 2013; Sarmah et al., 2006). Some antibiotics metabolize better than others, but excessive use of antibiotics results in antibiotic compounds passing through the gut unchanged and still active in human and animal waste (Kümmerer, 2009). These antibiotic residues exert a selective pressure on antimicrobial resistance (Sarmah et al., 2006). The antibiotic residues and ARGs present in animal and human waste are then introduced into aquatic environments through multiple pathways. Surface water runoff, wastewater treatment plant (WWTP) effluent, agricultural runoff, and even commercial fertilizers demonstrate human and animal waste as a pollution source of ARGs (Cira et al., 2021; Michael et al., 2013; Xu et al., 2015). The ocean may also be an important reservoir for ARB and ARGs. Globally, humans rely

on the ocean for food, recreational uses, and more, but the relationships between pathogens present in coastal environments and human health have not been fully established (Stewart et al., 2008). Furthermore, while ARGs and ARB are an emerging contaminant, monitoring efforts for antibiotic resistance have yet to be standardized, a necessary step before they can be widely implemented. Currently, there is uncertainty over the best methods for monitoring antibiotic resistance in the environment. The options include culture-, qPCR, and metagenomics-based techniques. A critical gap in our knowledge includes the relationships between the various methods for evaluating environmental antibiotic resistance and their connection to human health risk. As a result of the lack of standardization of methods, data quantifying levels of antibiotic resistance in different aquatic environments are not widely available. Thus, quantifying the amount of ARGs present in ocean water, particularly in surface coastal waters where humans are more likely to encounter any harmful pathogens, is pivotal to understanding and mitigating the threat of antibiotic resistance in aquatic environments.

The method of quantifying ARGs in surface oceanwater samples is important. The most widely used methods for analyzing ARGs are culture-based methods, quantitative Polymerase Chain Reaction (qPCR), metagenomics, and whole genome sequencing (WGS). Of these methods, a recent review by Franklin et al. on the different molecular methods of analyzing antimicrobial resistance in surface water categorized qPCR as the method that most precisely quantifies ARGs in different environments (2021). The study concludes that qPCR analysis is best used for assessment and comparison of ARGs and related genes within a study system (Franklin et al., 2021). Quantifying data is of particular importance because it can shed light on the factors that influence ARG levels. For example, quantifying surface water samples in dry and wet weather

can reveal whether rises in ARG levels detected coincide with runoff from certain sources into the oceans. Without a monitoring system, such quantitative data are not easy to locate.

As of the time this review was conducted, there were no published literature reviews regarding quantifying ARGs in coastal beaches. A review from Nappier et al. investigates antibiotic resistance in recreational waters (2020). Upon identifying potential sources of antibiotics, ARBs, and ARGs in recreational waters, the review concludes that monitoring the prevalence, concentration, and location of resistant bacteria are key to begin developing human health risk assessments and prioritizing the antimicrobial resistance risks to human populations (Nappier et al., 2020). Similarly, another review on the emergence of ARBs from coastal environments finds that more data is needed for a proper risk assessment of resistant bacteria in the environment (Jalal et al., 2012). To provide insight into what quantifications of ARGs are available and what is still needed, this systematic review will investigate the available literature that identifies at what levels ARGs are present in surface waters at coastal beaches.

Methodology

A systematic review was conducted using the Web of Science database to find published research papers that use qPCR analysis to quantify antibiotic resistant genes in surface water in coastal areas. The goal of the review was to compile available data and provide insight into the need for standardized methods for the risk assessment of antibiotic resistance in coastal areas.

Search words entered in the Web of Science database included Topic = ("antibiotic resistan*" OR "antimicrobial resistan*") AND Topic = (coastal OR ocean OR seawater OR "marine water" OR beach OR "recreational beach") AND Topic = (*PCR OR "* polymerase chain reaction"). These results were exported into a spreadsheet where each paper was categorized according to

the exclusion criteria. To find quantitative data for ARGs present in samples, any papers that did not use qPCR were excluded. The reason for this criterion is to understand how many studies quantify ARGs and what results stem from such analysis. Additionally, as mentioned before, there are varied methods for analyzing ARGs in samples so it is necessary to eliminate papers that use PCR, which would be included in search results but not needed for this review since PCR analysis provides information on the presence of ARGs but not the amount. Another exclusion criterion for the systematic review included eliminating research papers that studied aquaculture systems. This criterion was chosen because ARGs present in aquaculture systems are influenced by antibiotics used in fish farming, whereas this review is focused on risk assessment of ARGs in coastal waters accessible to the public. The third criterion was for papers that did not include surface water sampling in coastal areas. This criterion was included to eliminate studies that analyze sediment and animal samples, or any studies on the deep ocean. Like the second criterion, this criterion is to focus this review on the coastal environment accessible to the public. Lastly, any papers that were not related to antibiotic resistance but were included in the Web of Science search results were excluded. A summary of the papers excluded by each criterion is provided in Table 1.

Table 1: Summary of exclusion criteria and the corresponding number of papers excluded.

Exclusion Criteria	Number of Papers Excluded
All papers that do not quantify ARGs by qPCR	136
All papers that involve aquaculture systems	22
All papers that do not sample surface oceanwater	72
Papers not related to antibiotic resistance work	5
Total papers included in search results	181
Total papers fulfilling all criteria	12

The abstract, sampling methods, and methods of analysis were identified to determine whether each paper provided the desired quantitative data. Notes were taken, summarizing what sampling

was done and what data was provided. Each paper that surpassed the set criteria was thoroughly read and compiled into another spreadsheet. Here, the type of ARGs targeted for qPCR and the results of qPCR were extracted from the papers. In papers that provided qPCR results in the form of graphs, values were carefully estimated. Absolute abundance data was graphed (Figure 1). For multiple samples for the same location, gene counts were averaged.

Results and Discussion

Systematic Review

Of the 181 search results from Web of Sciences, a total of 12 papers were not excluded from the results and were closely reviewed. There were four papers that included qPCR analysis and coastal water sampling, but those samples were not the focus of the research study. The paper by Hou et al. was centered on antibiotic resistance in urban ponds but included 14 coastal seawater samples as natural reference for ARG levels (2020). The authors concluded that urban ponds are hotspots for the spread of ARGs. Notably, the authors also identified indicator ARGs for tracing the impacts of ARGs in the influent and effluent of a wastewater treatment plant (WWTP) and in ponds. The research study by Xiang Li et al. looked at qPCR results against MST microarray results from a variety of water samples, including two marine water samples (2016). The authors concluded that while microarray was correlated with qPCR quantification, additional culture-based or qPCR methods would be necessary to affirm the link between human health outcomes and pathogen gene detection through microarray (Li et al., 2016). The Ng et al. study was focused on the microbial composition and diversity of the open ocean and coastal environments (2015). The sampling involved three surface water samples in a harbor to compare against water samples from the ballast tanks of ships in the harbor. Lastly, the Suzuki et al. research paper

gathered four coastal seawater samples for an assessment of the detection of sulfonamide resistant genes (*sul1*, *sul2*, and *sul3*) in cultured sulfamethoxazole-resistant bacteria (SMX^r) and the natural bacterial assemblage in the water samples (2013). Results of the analysis suggest that *sul3* genes in non-culturable bacteria are associated with the marine environment, while *sul1* and *sul2* were detectable in each bacterial assemblage. The authors suggest that non-culturable bacteria can reveal different hosts in varied environments as potential reservoirs of ARGs in natural environments.

Table 2: Author name, sampling methods used in study, results presented, and data provided for papers closely reviewed.

Source	Sampling methods	Results Presented (ARGs)	Data provided
Carney et al.	Weekly, two-year duration time series.	*dfrA1 qnrS sul1 tetA vanB*	Absolute abundance (gene copies/L)
Chen et al.	Samples taken during dry and wet weather.	*tetB tetC tetM tetO tetW*	Absolute Abundance (gene copies/mL water)
Hou et al.	Seawater samples taken as background for ARGs present in urban ponds.	-	Absolute abundance (gene copies/ mL)
Jang et al.	Sampling before, during, and after two storm events	*tetD tetB ermB tetZ tetQ* bla_{TEM} *tetX aac6 sul1*	Relative abundance (gene copies/mL)
Li et al. (2016)	Samples used to evaluate the potential for microarray to detect pathogens.	-	-
Li et al. (2020)	Two sampling periods to compare wet and dry season, peak tourism and tourist off-season.	*cmlA floR qnrA qnrD qnrS sul1 sul2 tetG tetX tetW int1*	Absolute abundance (gene copies/ mL)
Ng et al.	Samples taken for comparison of resistance profiles in ballast water tanks and surrounding seawater at a bay.	*drfa sul1 ereA ereB ermB1 ermC ermF1 ermG ermT1 tetO tetM cfr*	Absolute abundance (gene copies/ mL)
Suzuki et al.	Samples used for distribution profiles in colony forming bacterial assemblages and natural bacterial assemblages.	*sul1 sul2 sul3*	Relative abundance (gene copies/16S)
Uyaguari et al. (2011)	Samples taken from a WWTP and surrounding water.	bla_{M-1}	Abundance (gene copies/mL water and gene copies / 16S)
Uyaguari et al. (2013)	Samples taken for comparison of resistance profiles in WWTP effluent and coastal waters in urban and non-urbanized areas.	*int1 int2 int3*	Abundance of integrons (gene copies/ mL and gene copies/ ng 16S)
Xin et al.	Sampling points taken on the same day, at differing distances from land.	bla_{OXA-58}	Relative abundance (gene copies/16S)
Zhang et al.	Samples differentiated by intracellular and extracellular DNA.	*sul1 sul2 tetM tetB* bla_{TEM} *qnrS*	Relative abundance (gene copies/16S)

Absolute abundance of ARGs

Of the papers that presented absolute abundance data, five research studies presented qPCR results for certain targeted ARGs. These values were extracted and compiled into Figure 1. The study by Carney et al. did a two-year sampling set for five different genes plus *int1* measurements. While the qPCR results were not easily extracted nor provided in supplementary documents, the results of the study are impactful. By combining culture methods with qPCR, the authors were able to provide strong evidence of the link between stormwater runoff from an urbanized area and the occurrence and persistence of ARGs present in urban coastal habitats. This study echoes the type of assessments other authors like Franklin et al. and Li et al. recommended for better risk assessments of ARGs in aquatic environments.

The eight most common ARG types are aminoglycoside, beta- lactamase, chloramphenicol, Macrolide-Lincosamie-Streptogamin B (MLSB), multidrug, sulfonamide, tetracycline and vancomycin (Hou et al., 2021). While there was not enough data to identify any trends, Figure 1 shows that the Jang et al. study measured high abundances for the sulfonamide, tetracycline, and aminoglycoside genes (2021). The Ng et al. study also found high abundance for tetracycline resistant genes (2015). Comparing the common ARG types found in studies with the most prescribed antibiotics in the study areas can provide an additional link between runoff into the oceans and rising resistance in coastal waters. Additionally, knowing the concentrations ARGs in an area can reveal any pollution by raw or treated wastewater discharged into the ocean from nearby cities, as the Hou et al. study discovered (2020).

Data Comparability

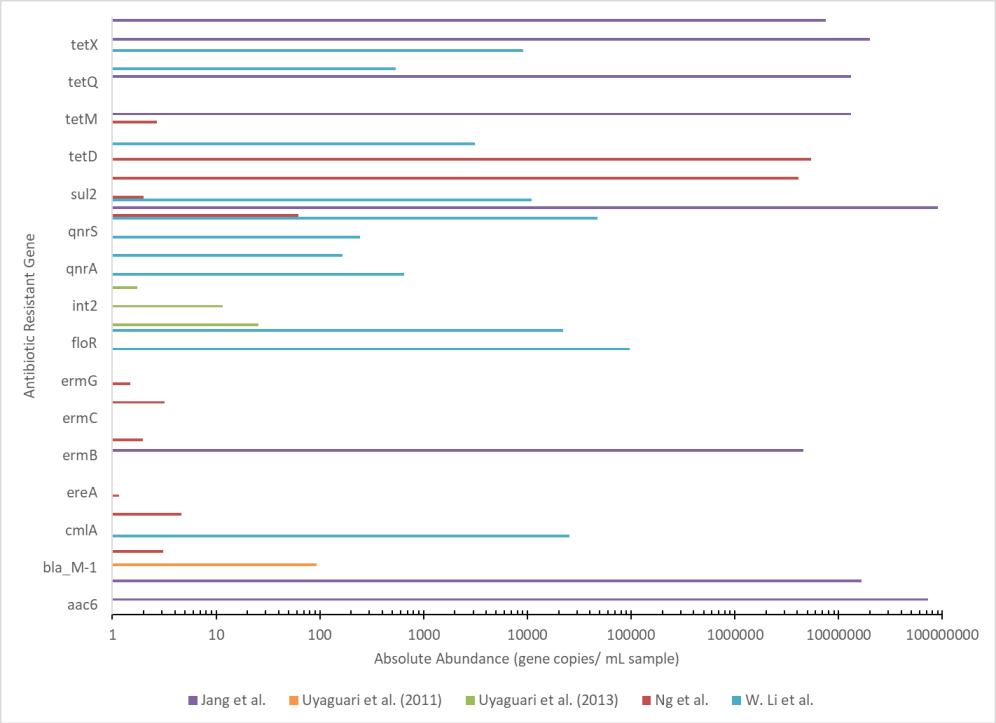

Figure 1: Abundance data for specified ARGs found in literature. A total of five studies were used.

The lack of comparable data found from this review highlights the need for a more standardized method of reporting ARGs in different environments. More papers reported qPCR results in absolute abundance, allowing for the compiling in Figure 1 to be done. However, when attempting to compile papers that reported relative abundance data, it became clear the data sets were not comparable. Each study either focused on a specific gene, did not present the data, or the data was not easily extractable or provided. In addition, the many different genes that can be detected by qPCR analysis lends to targeted gene variability between studies. This is evident in Figure 1, where only four genes were detected in more than one study location. This is likely a result of gene concentration varying by location, but if a monitoring system were to be

implemented, it would be necessary to distinguish which genes were below detection limits and which were not a targeted gene by qPCR.

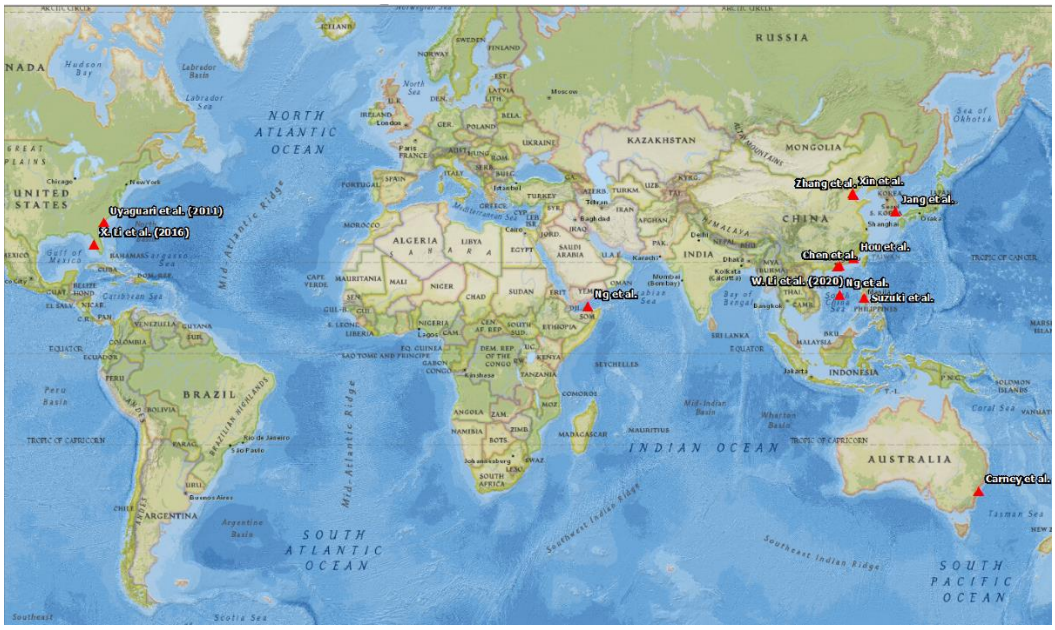

Figure 2: Sampling locations of the twelve papers reviewed, marked by pushpins.

Eight of the studies reviewed sampled in the China Sea, off the coast of China, Korea, and the Philippines. Three studies took samples in the Atlantic Ocean, off the coast of the United States. One study sampled in the South Pacific Ocean, off the coast of Australia. When attempting to compare the data from the eight studies in the South China Sea, only three studies reported which genes were targeted by qPCR analysis. This further highlights the need for standardized methods for reporting qPCR results, as the eight clustered cities could have provided insight into abundance trends in the South China Sea.

Implications for Future Studies

In summary, the results of this review revealed the following:

- There are not enough quantitative data for ARGs in coastal waters to identify any trends.
- A standardized way of reporting qPCR results would make data comparable for studies in different areas.
- Studies that sample over a longer time series can reveal trends in ARG concentrations.
- Further analysis is needed to determine the public health risks of antibiotic resistance in coastal environments.

Conclusions

The goal of this systematic review was to determine how many papers available through the Web of Science database provided qPCR results for surface coastal water sampling. Of the 181 search results, 169 papers were eliminated either because the paper did not involve antibiotic resistance work, did not use qPCR analysis to quantify ARGs, did not sample surface coastal waters, or the paper involved resistance within aquaculture settings. While the intent was to provide insight on the presence of ARGs in coastal environments based on compiled quantitative data, much of the data within the 12 papers closely reviewed were reported in different units, different targeted genes, or the samples measured were not the focus of the study. Ultimately, this review demonstrates the need for a standardized reporting of the concentration of ARGs. Once standardized methods are established, data reported by different studies would be comparable and trends can be clearly established for risk assessments. Additionally, the results of this review exposed the need for more quantitative data on ARGs and ARBs in the ocean. Such quantitative data would corroborate studies that suggest links between antibiotics and ARGs present in

stormwater runoff, agricultural runoff, wastewater treatment plants, and hospital effluents and increased resistance in the ocean. With the number of antibiotic resistant infections reported in the U.S. alone, knowing whether antibiotic resistance is concentrated in certain environments could help improve mitigation efforts.

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Chapter Two – Quantifying antibiotic resistant genes in two Southern California beaches

Introduction

The fate and transport of antibiotic resistance genes (ARGs) in the environment contributes to the global rise in antibiotic resistant infections. ARGs are the genetic material that encode defense mechanisms that render antibiotics ineffective at treating or killing harmful bacteria (Reygaert, 2018). ARGs can intrinsically be present in bacteria and can spread between bacteria through horizontal gene transfer. Extensive research has been done on ARGs and antibiotic resistant bacteria (ARB) to determine their dissemination in different aquatic environments. The excessive use of antibiotics in humans and animals results in antibiotic compounds passing through the gut unchanged and still active in human and animal waste, where they further disseminate into different environments (Kümmerer, 2009). Surface water runoff, wastewater treatment plant (WWTP) effluent, agricultural runoff, and even commercial fertilizers demonstrate human and animal waste as a pollution source of ARGs (Cira et al., 2021; Michael et al., 2013; Xu et al., 2015).

In Los Angeles, runoff from various sources and wastewater effluent drain to the ocean. Additionally, much of the Southern California coast is open for recreational uses. Understanding the health risks of exposure to impaired waters can potentially lower the number of antibiotic resistant infections. To investigate whether the concentration of ARGs in coastal areas can reveal any resistance trends and provide insight into ARGs as a threat to public health, coastal water samples from two Southern California beaches, Venice beach and El Porto beach, were analyzed. The data in this paper is from a larger project paper by Megyn Rugh (2021).

Currently, there is uncertainty over the best methods for monitoring antibiotic resistance in the environment. The options include culture-, qPCR, and metagenomics-based techniques. As a

result of the lack of standardization of methods, data quantifying levels of antibiotic resistance in different aquatic environments are not widely available. As such, the goal of this study is to provide quantitative qPCR data on ARGs present in Southern California ocean water to help understand and mitigate the threat of antibiotic resistance in aquatic environments.

Methodology

Sampling Methods

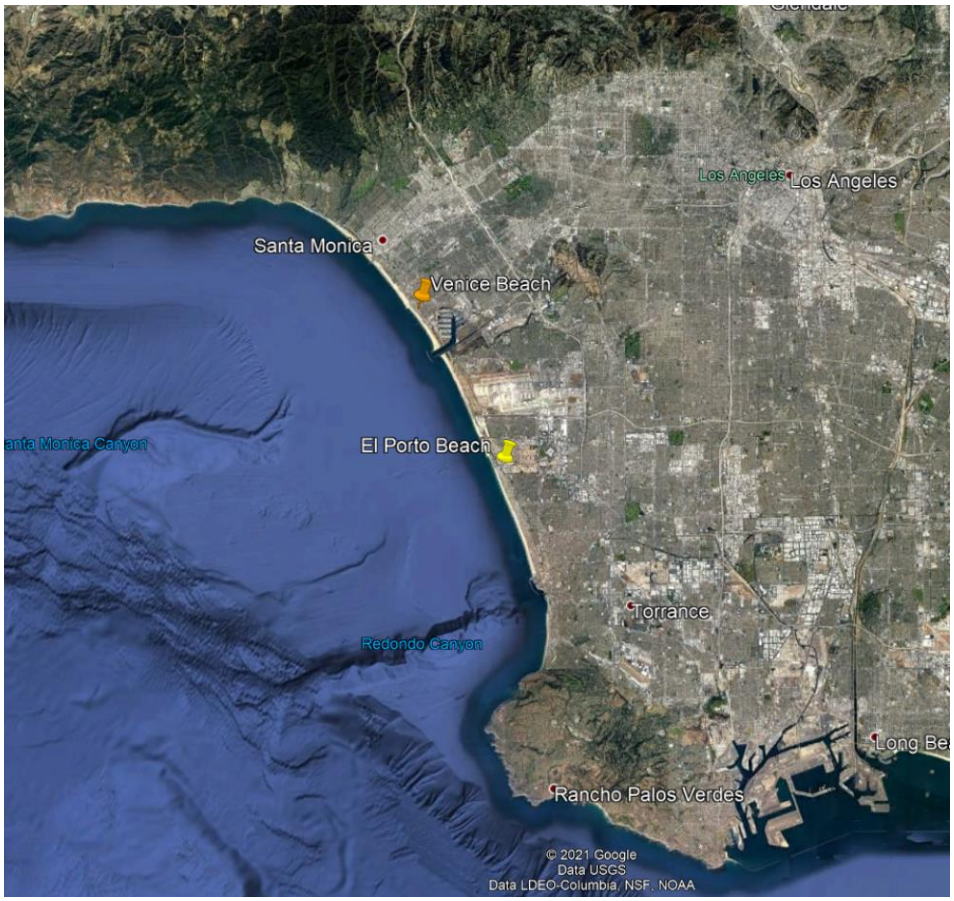

Figure 3: Image from Google Earth showing the locations of Venice and El Porto beaches in Southern California.

El Porto and Venice beach are located along the Southern California coast. Both beaches are open for recreational uses and experience high foot traffic. This region of California does not get

heavy rainfalls. Precipitation data for the duration of this study is demonstrated in Figure 4, adapted from the National Centers for Environmental Information (NOAA). Bacterial populations are known to spike due to the fist flush effect, where the accumulated bacteria on land are washed away in the beginning of a rain event (Ackerman & Weisberg, 2003). Therefore, surface water samples were collected in both wet and dry weather conditions.

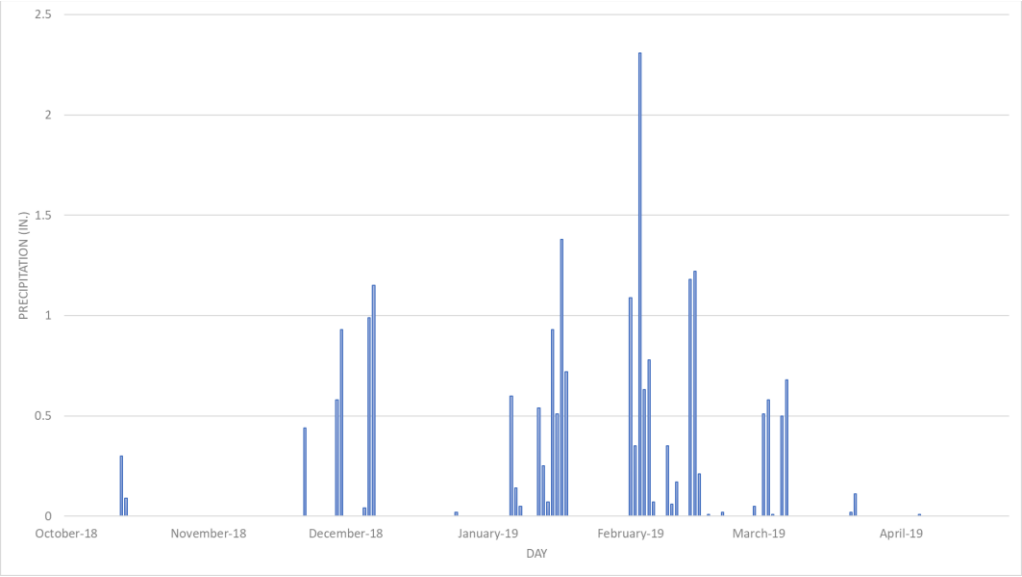

Figure 4: Precipitation data for 2018-2019 during sampling time series.

From October 2018 to April 2019, triplicate samples were collected at both beach sites. Marine water samples were collected in sterile 2L Nalgene. Surface ocean water was collected specifically during an incoming wave. Containers were thoroughly rinsed with sample water immediately prior to sample collection. Samples were stored on ice and transported to the laboratory within two hours of collection and filtered within three hours of arriving in the laboratory.

Quantitative PCR

The following section is adapted from Megyn Rugh's project paper (2021). The targeted genes chosen for analysis by qPCR were macrolide-lincosamide-streptogramin resistant gene *ermB* , sulfonamide resistant genes *sul1* and *sul 2*, beta-lactamase resistant gene bla_{SHV}, integron *int1*, and the 16S rRNA gene. A summary of the primers used are provided in Table 1. Amplification for all genes excluding 16s rRNA gene was performed in a StepOnePlus (Applied Biosystems, Foster City, CA) in 20 μL reaction mixtures on 96-well plates containing PowerUp SYBR ® Green Master Mix (Applied Biosystems, Foster City, CA), 4μL aliquots of template DNA, and forward and reverse primers at 200nM final reaction concentration. For each plate a seven-point standard curve was used along with negative control. The 16s rRNA assay was performed in 25 μL reaction mixture in on 96-well plates containing PowerUp SYBR ® Green Master Mix, 2 μL aliquots of template DNA, and forward and reverse primers at 600nM final reaction concentration. For each plate, a five-point standard curve was used along with negative control. Each sample was performed in triplicate wells, from which the standard error was calculated.

Gene	Forward / Reverse Primer	Amplicon Size (bp)
sul1	CGCACCGGAAACATCGCTGCAC/ TGAAGTTCCGCCGCAAGGCTCG	22/22
sul2	CTCCGATGGAGGCCGGTAT/ GGGAATGCCATCTGCCTTGA	19/20
blaSHV	TGATTTATCTGCGGGATACG/ TTAGCGTTGCCAGTGCTCG	20/19
int1	GGCTTCGTGATGCCTGCTT/ CATTCCTGGCCGTGGTTCT	19/19
ermB	AAAACTTACCCGCCATACCA/ TTTGGCGTGTTTCATTGCTT	20/20
16S rRNA	ATGGCTGTCGTCAGCT/ ACGGGCGGTGTGTAC	16/15

Table 3: Primers used for qPCR in this study.

Results and Discussion

Precipitation and abundance

There were 40 timepoints from Venice beach and 38 from El Porto beach. Samples were collected during wet weather and dry weather conditions. Figure 5 depicts the absolute abundance in samples throughout the time of the study, with the corresponding precipitation provided on a second axis. The wet weather season in Los Angeles is typically between November to March. ARG spikes were demonstrated following rain events, particularly in the months of December and January. Even with timepoints that were not exactly on the first day of a rain event, spikes were evident. For example, between January 12 and January 18, precipitation was recorded on each day. Surface water samples were taken on January 15 and January 22. Spikes in ARG concentration were detectable on the 15th, but not on the 22nd, following the rain event. A similar trend was seen from February 1 to February 6, where sampling was done once during the final day of the rain event. No spike was detected in this case. This corroborates the first flush effect, as previously mentioned. The bigger implication of this trend is the suggestion that the ARGs present in the ocean may be a result of anthropogenic influences. A similar study by Carney et al., which sampled over a period of two years at a beach in Australia, found evidence of a link between stormwater inputs from an urban watershed and the occurrence and persistence of ARGs (2019).

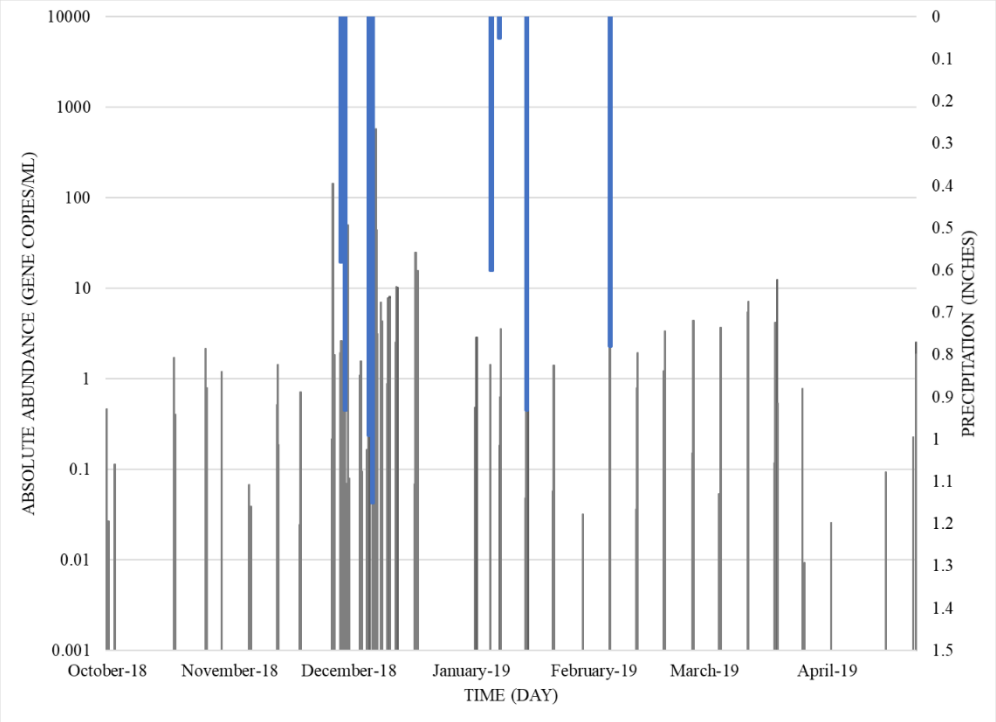

Figure 5: Absolute abundance and precipitation plotted over the course of the study timepoints.

ARG levels detected

Abundance data for each site are shown in Figures 7 and 8. From the four ARGs targeted by qPCR analysis, *sul2* showed higher abundance values in samples, followed in order by *sul2*, *ermB*, and bla_{SHV}. At Venice beach, *ermB* abundance ranged from 0.02 gene copies/mL to 7 gene copies/mL, *sul1* abundance ranged from 0.06 gene copies/mL to 49.7 gene copies/mL, *sul2* abundance ranged from 1.95 gene copies/mL to 142.88 gene copies/mL, and bla_{SHV} abundance ranged from 0.001 gene copies/mL to 3.15 gene copies/mL. At El Porto beach, *ermB* abundance ranged from 0.01 gene copies/mL to 0.44 gene copies/mL, *sul1* abundance ranged from 0.09 gene copies/mL to 49.7 gene copies/mL, *sul2* abundance ranged from 0.79 gene copies/mL to 1127.23 gene copies/mL, and bla_{SHV} abundance ranged from 0.01 gene copies/mL to 0.18 gene copies/mL.

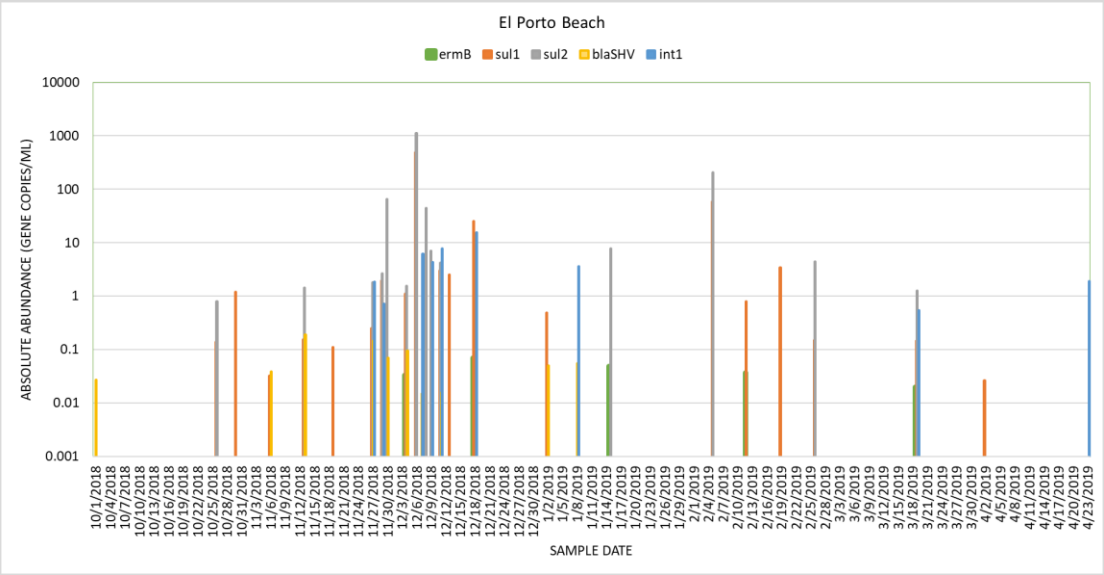

Figure 6: Abundance data for El Porto samples.

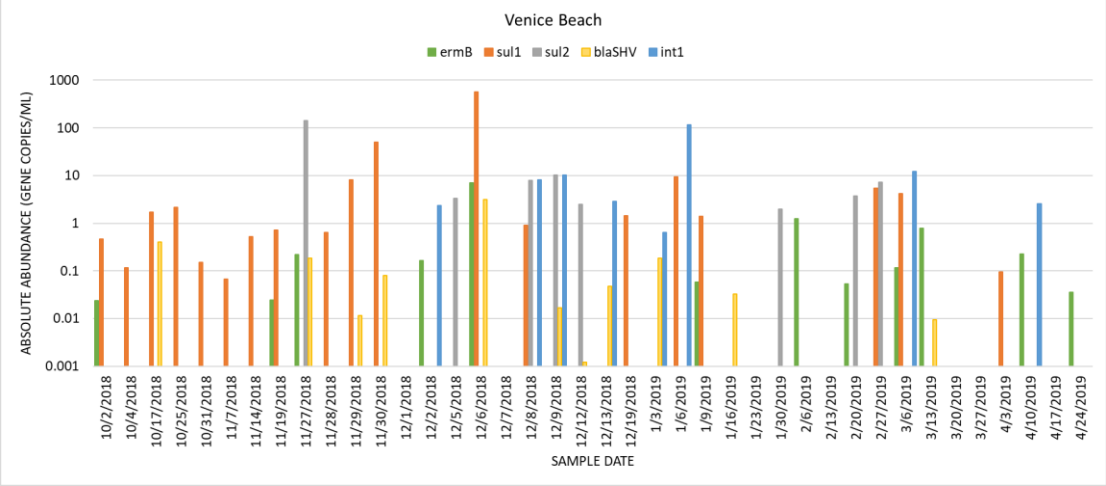

Figure 7: Abundance data for Venice samples.

Conclusion

Surface water runoff and wastewater treatment plant (WWTP) effluent are each a known pollution source of ARGs. In cities like Los Angeles, where most surface runoff and WWTP effluent are being drained into the ocean, it is important to understand the impact such practices have on aquatic environments. While previous studies have established the need for ARG monitoring, few studies have quantified ARGs in coastal areas. This study sampled from two Southern California beaches to provide quantitative ARG data and identify any potential trends of ARGs present in coastal waters. As reported by a similar study, wet weather and dry weather sampling revealed the impact of stormwater inputs and peak ARG concentrations in the ocean.

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